Clinical and biological markers of progression in Alzheimer's disease

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ABSTRACT

Rates of disease progression differ among patients with dementia due to Alzheimer's disease (AD), but little is known about prognostic predictors. A number of clinical, neuroimaging and biochemical factors have been found to increase the risk of elderly individuals developing AD. However, little is known whether such factors also play a role in the progression of the dementia itself. The aim of the study was to assess predictors of cognitive decline in people with dementia due to AD using different strategies in longitudinal studies. In a clinical study, smell identification test was used to evaluate its utility as clinical marker of progression. Higher smell identification dysfunction indicated more severe disease with lower cognition, higher functional dependence and more behavior symptoms. Although the baseline UPSIT scores were associated with baseline MMSE scores, it did not interact significantly with change in MMSE over the follow up period. In parallel, another complementary approach was used to examine if certain plasma proteins predict illness progression. Plasma proteins clusterin and transthyretin were found independently, to be associated with disease severity and progression. Lastly, MRI structural brain measures were examined for progression markers and established entorhinal cortex thickness predicted cognitive decline. Together the studies provide evidence that non-invasive techniques such as clinical olfaction assessment, blood tests and structural MRI can measure disease severity and that blood proteins and MRI brain measures can predict cognitive decline in mild-moderate AD dementia.

Non-invasive markers of progression are of great importance to accurately define subgroups that may differ in rate of progression, presumably reflecting different stages of expression of Alzheimer neuropathology, may be useful in understanding underlying AD mechanisms and predicting therapeutic response. The thesis studies present interesting candidates; however, external replication is essential for establishing these markers. Further studies are needed to test their value and utility in biomarker panels for improving prediction, diagnosis and monitoring of AD.

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DEDICATED TO MY SON, SANKALAN

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CHAPTER 1

INTRODUCTION

1. INTRODUCTION

As a clinician I am frequently asked by patients newly diagnosed with dementia due to Alzheimer's disease (AD) and their families, 'How severe is the disease? Will it get worse? How much longer before it gets bad? ' The ability to predict progression rates in Alzheimer's dementia would aid clinicians, patients and families for the long-term planning of care and treatment of patients. Better predictive markers would also be important for clinical trials to assess the efficacy of disease-modifying therapies.

AD is progressive and the commonest form of dementia, responsible for 60 to 80 percent of all dementia. Alzheimer's disease is characterized by gradual decline of memory and other cognitive functions, in addition to progressive loss of daily functioning and associated neuropsychiatric symptoms (APA, 1984). Each year 39,400 more people are diagnosed with Alzheimer's disease in England and Wales; that equates to one new case every 14 minutes (Copeland et al., 1999). As a consequence of an aging population, the prevalence of AD is set to rise in coming decades (Mashta, 2007), resulting in a huge financial and emotional burden for society and for caregivers.

AD likely begins histopathologically many years before clinical symptoms manifest (Braak and Braak, 1991). The insidious onset of symptoms makes it difficult for the patient and family to be definite when the abnormal cognitive or functional state actually started and patients therefore come to a medical diagnosis at variable intervals after the first symptoms begin (Doody et al., 2001). Previous studies have reported average estimates of disease duration from 3 to 4.5 years at the first clinical presentation (Kraemer et al., 1994, Morris et al., 1993, Bracco et al., 1994). A period of symptomatic disease often exists at the time of presentation, allows an estimate of initial rates of decline, that is; relatively predictive of subsequent disease progression (that is initial rapid progressors will continue to decline faster than the initial slow progressors) (Doody et al., 2001, Doody et al., 2010). Natural history studies and data on placebo groups from drug trials have reported tremendous heterogeneity between-subjects and between-groups of measured progression rates, which reflects multiple phenomena, including, 1) true difference in disease progression rates between patients,

2) differing properties, i.e., floor and ceiling effects, of the measures used, 3) differences in the end point selected to represent progression (cognitive decline, functional decline, nursing home placement, or death), 4)other methodological differences, such as the number of patients, duration of follow up and interval between visits, 5) differences in medical comorbidities, and 6) differences in patient care (Doody et al., 2001). Some authors have suggested that the initial stage of disease at the beginning of the observation period ("how far") is an important predictor of subsequent decline ("how fast") (Kraemer et al., 1994). Most staging measures fail to document linear decline over the course of AD and as a result both 'bilinear' and 'trilinear' models of decline have been proposed (Morris et al., 1993, Stern et al., 1994, Brooks et al., 1993, Doody et al., 2001). However, no clear, predictive models of progression have been developed and validated for AD. Baseline cognitive function has been shown to correlate with future cognitive decline (Sona et al., 2012). Among several studies which investigated clinical predictors in AD, some authors have associated extrapyramidal signs, severe cognitive impairment at baseline and high preprogression rates (PPRs) with rapid decline (Schmidt et al., 2011). The concept of PPRs introduced by Doody describes the rate of cognitive decline before the time of diagnosis (Doody et al., 2001). Hereby, the difference of the Mini Mental State Examination (MMSE) of healthy contemporaries at the time of clinical onset and the MMSE at diagnosis is divided by the time span between onset of AD and diagnosis (Doody et al., 2001). Nevertheless, this was studied in a single patient group (n=298). A recent study which re-examined Doody's hypothesis that PPRs predict short-term decline of AD patients (n=78) within the first year after diagnosis in a different cohort and found that PPRs were associated with the decline of instrumental activities of daily living (iADL) but not MMSE decline (Schmidt et al., 2013).

Disease progression is heterogeneous; the rates of decline in cognitive and functional capacities are variable, and there are different rates of institutionalization and death between patients (Cortes et al., 2008). The factors that influence or predict progression are not well understood (Kraemer et al., 1994, Marra et al., 2000). Disease progression is measured, most commonly, by change in cognition over time (Kraemer et al., 1994, Marra et al., 2000). However, clinical and neuropsychological measures may lack sensitivity to change, are subject to day-to-day variability, and are influenced by

behavioural fluctuations and inter-current illness and medications (Sluimer et al., 2008).

The purpose of this study is to identify disease progression markers for dementia due to AD. While there are potentially various kinds of markers such as neuropsychological, neuroimaging etc that may be used to predict disease progression in AD, I attempted to identify markers using complementary approaches that may, if successful, help in prognostication using simple inexpensive tests. Hence, one of the approaches that I have taken is to investigate whether a simple bedside clinical measure of olfaction could predict disease progression. Another complementary approach that I have taken builds on my previous gel based proteomic discovery work and involves examining if certain plasma proteins predict disease progression in AD. Lastly, examined MRI brain structural regions for markers of cognitive decline in patients with AD dementia.

2. Objectives:

1. To test whether smell identification test can be used as a predictor for illness progression in patients with mild-moderate AD dementia and to study the rate of smell dysfunction with illness progression.
2. To test if there is difference in plasma protein concentrations between the rapid and non-rapid progressors and if this predicts the progression of illness.
3. To examine the relationship between baseline hippocampal volume, entorhinal cortex thickness and whole brain volume with baseline cognitive measures and with subsequent cognitive change over one year period.

2.1 Olfactory dysfunction in general and impaired odour identification in particular have been reported in AD and occur at early stages of the disease (Mesholam et al., 1998). Odor identification tests have demonstrated high sensitivity and specificity in discriminating AD patients from controls (Morgan and Murphy, 2002), wherein 12 patients with AD and 12 matched healthy controls were tested for smell identification and olfactory event related potential. Combination of the odor identification scores with olfactory P3 latency measures resulted in a correct classification rate of 100%. Smell identification test has also been studied as a marker for predicting conversion from mild

cognitive impairment (MCI) to AD (Devanand et al., 2008, Devanand et al., 2000). This longitudinal study followed up 148 outpatients with MCI over 3 year period and found smell identification as one of the 5- predictors when combined strongly predicted conversion to AD. There has been a proposal to include olfactory dysfunction within the diagnostic criteria of AD (Foster et al., 2008). It has been indicated that involvement of the olfactory bulb and tract is one of the earliest events in the degenerative process on the central nervous system in AD in an autopsy study of 110 cases- 91 AD cases and 19 controls (Christen-Zaech et al., 2003). Tau pathology in the olfactory bulb increases has also been shown to increase with severity of AD (Attems et al., 2005), suggesting a possible link between measures of olfaction and disease progression in AD.

I have previously investigated the utility of smell identification test as a predictor of response to treatment with cholinesterase inhibitors in patients with AD dementia (Velayudhan and Lovestone, 2009) The current study further extends this work to examine whether performance in a smell identification test can be a reliable marker of disease progression in AD. Olfactory dysfunction has not been investigated as a progression marker, although it has been studied as a diagnostic marker as well as a marker of conversion in AD. As this is an inexpensive, brief test that is easy to carry out in a clinical setting with minimal training, it may have application in clinical settings in adjunct with other measures in monitoring the progression of AD.

This part of study has been described in detail in Chapter 3: 'Smell identification test as a marker for severity and progression in AD'.

2.2 Plasma protein markers:

Studies have evaluated biochemical diagnostic markers in both cerebrospinal fluid (CSF) and blood in AD. Because of the invasive nature of lumbar puncture for obtaining CSF, particularly in elderly people, biomarkers that are detectable in blood, which is more easily obtained, are considered more practicable in a clinical setting. Recently, progress has been made in identifying plasma proteins as potential diagnostic biomarkers for AD and for differentiating from other dementias, using proteomics technologies (Hye et al., 2006, Lovestone et al., 2007b, Song et al., 2009). Using filter-

based sandwich ELISAs, a panel of 18 plasma signalling and inflammatory proteins was identified from screening 120 known signalling proteins (Ray et al., 2007). Combined multivariate analyses of this panel of proteins enabled the accurate identification of AD patients and predicted the progression to AD in individuals with MCI. This has raised the possibility of investigating whether plasma proteins may also serve as markers of disease progression in AD (Lovestone et al., 2007b, Song et al., 2009).

The accumulation of the amyloid-beta (Aβ) peptide, the main constituent of the "amyloid plaque", is widely considered to be the key pathological event in Alzheimer's disease (Vardy et al., 2005). An increasingly detailed understanding of proteolysis in both Aβ deposition and clearance has identified some of these proteases as potential therapeutic targets for Alzheimer's disease. In addition to a number of in vitro studies, in vivo studies with amyloid precursor protein (APP) transgenic mice indicate that APOE and a related molecule, clusterin (also called apolipoprotein J), have profound effects on the onset of Aβ deposition, as well as the local toxicity associated with Aβ deposits both in the brain parenchyma and in cerebral blood vessels (Holtzman, 2004). Transthyretin (TTR) was the third protein found to interact with Aβ after apolipoprotein E (ApoE) and clusterin in AD CSF biomarker studies (Li and Buxbaum, 2011). TTR, a systemic amyloid precursor, can suppress Aβ aggregation in vitro and in vivo and ameliorate its pathologic effects in a well-validated transgenic mouse model of human AD (Li and Buxbaum, 2011).

In a pilot gel based proteomics discovery work, using 2DGE proteomic approach and LC/MS/MS, I identified potential plasma proteins that can serve as a disease progression marker in AD dementia. The plasma proteins identified were associated with immune regulation, inflammation, transporters of lipid, and also included Aβ binding proteins such as clusterin and TTR. The lab-based component of the present work involved testing clusterin and TTR proteins as AD dementia progression markers in independent and larger cohort.

The work published as part of the thesis is described in detail in chapter 4: ‘Plasma proteins as progression markers for AD’.

2.3. MRI neuroimaging markers

MRI has demonstrated significant value in the prediction of conversion and disease progression (Fennema-Notestine et al., 2009b). It has been shown that MRI is useful for detecting atrophy in the medial temporal structures affected early in the neurodegenerative process (Fennema-Notestine et al., 2009b). Numerous studies have used baseline and serial MRI measures to predict future cognitive decline but mostly for conversion from mild cognitive impairment (MCI) to AD (Varon et al., 2011, McEvoy et al., 2009, Cardenas et al., 2011, Mungas et al., 2001) and there is need for assessing these MRI measures as potential markers of disease progression in AD. Previous reports from European Union AddNeuroMed multi-center MRI study have shown that structural MRI measures discriminated AD from controls and MCI; and also demonstrated potential for prediction of conversion from MCI to AD (Costafreda et al., 2011, Liu et al., 2010b, Westman et al., 2011c, Liu et al., 2011a, Westman et al., 2011b, Westman et al., 2011a). My hypothesis was that smaller brain structures would be associated with worse baseline cognition and greater cognitive decline.

The paper now accepted for publication is described as chapter 5: Other AD progression markers: 'Entorhinal cortex thickness as marker of cognitive decline in AD'.

Summary

The present work is to examine whether different complementary approaches help in predicting disease progression in dementia due to AD. Although the approaches are distinct, they are bound together by their common theme of identifying disease progression markers in AD dementia that may have an application in clinical settings.

CHAPTER 2

OVERVIEW: DESIGN AND METHODOLGY

2. OVERVIEW: DESIGN AND METHODOLOGY

2. 1. Olfactory dysfunction as progression marker:

2.1.1 Subjects:

Alzheimer's disease dementia participants: Participants with late-onset, mild to moderate dementia due to AD (NINCDS-ADRDA criteria) (McKhann et al., 1984), were recruited and followed up through 2007-2011 (*n*= 39), from community mental health team (CMHT) within the mental health for older adults (MHOA) and through the Dementia Case Register, NIHR BRC IOP and South London and Maudsley (SLaM) NHS Foundation Trust. Patients were also recruited from the Charnwood Memory Service within the mental health service for older persons (MHSOP), Leicestershire partnership NHS Trust (2011-2012, *n*=25), where I am working now, following ethical committee approval for including it as an additional site.

Exclusion criteria: Dementia other than AD; history of psychiatric disorder, including substance abuse; medical conditions which affect olfactory functioning (e.g., blocked nasal passages, polyps etc.); current history of cigarette smoking.

Non demented controls (NDC): Eligible elderly volunteers as healthy controls were invited and recruited from other on-going research projects at the department of Old Age Psychiatry, Institute of Psychiatry (2009-2012, *n*= 28).

2.1.2 Study design and outcome measures

Cognitive testing with Mini Mental State Examination (MMSE) was used to classify AD patients into rapid and non-rapid decliners, a commonly used cognitive test to assess severity and decline in clinical settings (Behl et al., 2005). Those with a loss of 2 or more MMSE points at study inclusion, compared to their recorded score 6-months before will be classified as 'Rapid cognitive decliners', while the rest classified as 'Non-rapid cognitive decliners'.

Assessments were performed at baseline and at follow up after 3 months for both AD dementia and NDC groups i.e., Neuropsychiatric Inventory; Bristol Activities of Daily Living Scale; MMSE and olfactory identification function using the University of

Pennsylvania Smell Identification test (UPSIT). UPSIT is a standardised "scratch 'n sniff" test of odour identification.

2.1.3 Statistics

2.1.3.1 Power Calculation: A sample size of 24 in each group will have 80% power to detect a difference in means of 4.500 assuming that the common standard deviation is 5.400 using a 2-group t-test with a 0.050 two-sided significance level. The effect size from the given power calculation is 0.14. Although it is a small effect size, it would indicate 54-58% of a rapid decliner group below the average non-rapid decliner AD patient. This would still be substantial, given smell test is an easily applicable test in a clinical setting and it would be valuable as an adjunct with other tests. Also it would be feasible within the time-frame of this proposal.

2.1.3.2 Data analysis: Chi-Square, student's t-test and non-parametric Mann-Whitney tests were used for comparing the socio-demographic, clinical parameters (MMSE, NPI, UPSIT and BADL). Bivariate correlations were performed to determine correlation between MMSE, UPSIT, ADL and NPI scores and rapid and non-rapid decliners groups. Regression analyses were used to determine if baseline UPSIT measures predicted future MMSE.

2.2 Plasma proteins as progression markers:

In this study, initially gel based proteomics using 2DGE proteomic approach and LC/MS/MS was done to identify potential plasma proteins that can serve as a disease progression marker in AD dementia. The proteins were then tested in independent and larger cohort for its robustness using immunoblotting experiments.

2.2.1 Subjects and Samples:

The proposed study accessed a sample resource collected as part of the AddNeuroMed project, a European Union study cohort of AD dementia patients (AddNeuromed: http://www.innomed-addneuromed.com). As part of this study, individuals with mild-to-moderate AD dementia (NINCDS-ADRDA) have already been recruited and assessed. Following recruitment, they are assessed clinically and blood obtained at quarterly intervals over a year.

2.2.2 Pilot Work (Discovery phase):

I examined plasma samples from 51 subjects with mild-moderate AD dementia from the AddNeuroMed cohort, using a 2 Dimension Gel Electrophoresis (2DGE) proteomic technique. Patients were characterised as fast progressors based on a decline of 2 or more points on the Alzheimer disease assessment scale – Cognitive (ADAS-COG) score from baseline to the 6-month follow-up time point. Using this criterion, 22 subjects were characterised as 'rapid' and 29 as 'non-rapid' progressors. I identified 9 protein spots of interest following silver staining of the gels, image analysis using Progenesis same spot software (Nonlinear dynamics), and partial least squares discriminant analysis. Proteins from these spots which discriminated the two groups of patients were identified using LC/MS/MS.

2.2.3 Validation-phase (present work):

In the current project, I attempted to validate the proteins discovered from my previous study (described above), using specific assays, like Western blotting or Enzyme Linked immunoassay (ELISA), in an independent, larger patient sample (n~ 300). Relative and absolute quantification of the proteins in the different subject groups helped to establish their robustness as a marker.

2.2.4 Statistics:

SPSS 15.0 was used for statistical analysis of the data. Chi-Square, student's t-test and non-parametric Mann-Whitney tests were performed to compare the socio-demographic, clinical parameters (ADAS-cog, MMSE, NPI and BADL) and protein levels (µg/ml) between the groups. Bivariate correlations performed to determine correlation between protein levels and changes in cognitive measures (MMSE and ADAS-cog scores) from baseline to 6 months. Regression analyses were used to establish if baseline plasma protein levels predicted changes in cognitive measures.

2.3 Neuroimaging **markers of cognitive decline**

2.3.1 Participants, assessments and analysis

This study included data from participants from the AddNeuroMed study, a longitudinal, multi-centre European Union study, of biomarkers for AD

(AddNeuromed:http://www.innomed-addneuromed.com). As part of this study, subjects underwent MRI scanning at baseline and cognitive testing at baseline and every 3 months up to one year. Cognitive testing was done with Mini Mental State Examination (MMSE)(Folstein et al., 1975) and Alzheimer disease assessment scale – Cognitive (ADAS-cog) (Rosen et al., 1984) and stage of dementia with Clinical Dementia Rating (CDR) (Hughes et al., 1982) sum of boxes score.

These subjects had structural MRI data which was acquired as designed to be compatible with the Alzheimer Disease Neuroimaging Initiative (ADNI) (Jack et al., 2008). MRI was done within a month of the clinical/cognitive assessments and blood sampling. Freesurfer pipeline (version 4.5.0) was applied to the MRI images to produce regional cortical thickness and subcortical volumetric measures - hippocampal volume, entorhinal cortex thickness and whole brain volume.

Rates of cognitive decline were determined by change in the cognitive measures – MMSE and ADAS-Cog total scores. These measures were estimated by fitting a random intercept and slope model using xtmixed in STATA 10 (Stata Corporation, College Station, TX, USA). An interaction between the MRI-based brain volumes and follow- up time was used to indicate the association of baseline brain volume with baseline cognitive assessment score and the effect of brain volume on cognitive decline over time. The results were based on using the brain measures as continuous variables and the quartiles for graphical view.

CHAPTER 3

SMELL IDENTIFICATION DYSFUNCTION AS A SEVERITY AND PROGRESSION MARKER FOR ALZHEIMER'S DISEASE

ABSTRACT

Background: Factors influencing or predicting progression in dementia due to Alzheimer's disease (AD) is not well understood. Olfactory dysfunction, impaired smell identification in particular, is known to occur in AD. Mesial temporal lobe, important for memory function is also critical for the processing of olfactory information. In view of the common anatomical substrate for both memory deficits and the olfactory function in AD, this study aimed to evaluate the smell identification test for assessing illness progression in AD dementia patients.

Methods: Fifty seven outpatients with late onset mild to moderate AD dementia and 24 elderly non-demented controls (NDC) were assessed, at baseline and a follow up after 3 months, for mini mental state examination (MMSE), University of Pennsylvania Smell Identification Test (UPSIT), Bristol activities of daily living and Neuropsychiatry Inventory. AD dementia participants were classified as Rapid Cognitive Decliners (RCD) defined on 'a-priori' with a loss of 2 or more points in MMSE within the previous six months.

Results: AD dementia participants had lower olfactory scores than NDC. RCD had lower olfaction scores compared to Non-Rapid Cognitive Decliners (NRCD). Although the baseline UPSIT scores were associated with baseline MMSE scores, it did not interact significantly with change in MMSE over the follow up period. Using a median split for olfactory scores, the AD dementia participants were classified as Rapid Olfactory Progressors (ROP) (UPSIT ≤ 15) and Slow Olfactory Progressors correlating significantly with RCD/NRCD groups. The ROP group with higher olfactory impairment indicated more symptomatic illness or severity, i.e., lower cognition, higher functional dependence and presence of behavioural symptoms.

Conclusions: Our study supports association of smell identification function with cognition and its utility as an adjunct clinical measure to assess severity in AD dementia. Further work, including larger longitudinal studies, is needed to explore its value in predicting AD dementia progression.

3.1 BACKGROUND

Olfactory dysfunction in general and impaired odour identification in particular, have been reported in AD and are found to occur at early stages of the disease (Mesholam et al., 1998).

3.1.1 Evidence

3.1.1.1 Olfactory impairment in AD and other neurodegenerative disorders:

Olfactory impairments in Alzheimer's disease were first reported by in 1974 (Waldton, 1974) and it is now well established that those with AD have abnormal olfactory function.

The different tests for olfaction are:

Olfactory identification: In odor identification tasks an odorant is presented at a suprathreshold concentration and subjects are required to identify the odor from a list of descriptors. This forced-choice procedure controls the subjects' response bias.

Odor discrimination: Subjects have to determine which of the suprathreshold odorants smell different. Thus, they demonstrate the ability to distinguish between odors, not to recognize or identify them.

Odor fluency: Olfactory analog to verbal fluency tests (Bacon Moore et al., 1999) , in odor Category Fluency (OCF), exactly as in the verbal fluency test, participants are asked to generate as many things that belong to the category, *odors*, or things with odors, as they could within 60 seconds. Odor Letter Fluency (OLF), participants are told that they would be given a letter and asked to name as many *things with odors* as they could that began with the specific letter they were given. Finally, Odor Stimulus Fluency (OSF), participants are presented with an odorant stimulus and asked to name all of the odors that the stimulus brought to mind.

Odor recognition memory: Ability to verbally recall previously presented odors and to learn this task across trials (immediate and delayed recall both free and cued).

Odor threshold: The concept embedded in threshold tests is that a subject is repeatedly exposed to ascending and descending concentrations of the same odorant and is required to identify the least detectable concentration for this individual odor.

Olfactory dysfunction is also known in other neurodegenerative disorders such as, frontotemporal dementia, Lewy body dementia, Huntington's' dementia and Parkinson's disease. A meta-analysis concluded that impairments have been shown on tests of olfactory identification, olfactory recognition memory and olfactory threshold in both patients with AD and PD relative to controls (Mesholam et al., 1998). Patients with AD (n=40) and Huntington's dementia (n=11) had impaired odor fluency compared to age matched controls (Bacon Moore et al., 1999). Poor odour recall and recognition memory were found in patients with pathologically confirmed AD and Lewy Body variant of AD compared to elderly controls (Gilbert et al., 2004). Performance on tests of odour discrimination, naming, and matching was compared in patients with 4 distinct neurodegenerative disorders and, severe impairment was found in identification in semantic dementia and AD, with mild impairments in frontotemporal dementia and corticobasal degeneration (Luzzi et al., 2007).

A recent meta-analysis (Rahayel et al., 2012) which included 39 articles with AD and 42 with PD, suggests that AD even more than PD, affect more strongly odour identification and then odor detection. PD patients are more impaired on detection thresholds than AD patients. These results suggest that PD patients are more impaired on low-level perceptual olfactory tasks whereas AD patients are more strongly impaired on higher-order olfactory tasks involving specific cognitive processes.

3.1.1.2 Smell identification and AD

Severe deficits in odor identification in AD have been well documented (Doty et al., 1987, Morgan et al., 1995, Moberg et al., 1997, Chan et al., 2002). A recent meta-analysis shows that there are no published reports of smell identification in AD which have failed to find deficits relative to healthy elderly (Rahayel et al., 2012).

Odor identification and detection was tested in 55 patients with AD dementia and 57 elderly control subjects by using the University of Pennsylvania Smell Identification Test (UPSIT) (Serby et al., 1991). Significant deficits in olfactory identification were present in patients, and these deficits increased as AD progressed and smell identification test scores were correlated with Mini-Mental State scores. In a small study, combination of the odor identification scores with olfactory event related

potential latency measures resulted in a correct classification rate of 100% in 12 patients with AD and 12 matched healthy control (Morgan and Murphy, 2002). The UPSIT successfully differentiated between dementia patients (AD and vascular dementia, n=13 each) and normal elderly British subjects (n=13) tested in their homes (Gray et al., 2001). In a non-blind uncontrolled study, smell identification function was found to be useful as a clinical measure to assess treatment response with donepezil in AD dementia patients (n=25) (Velayudhan and Lovestone, 2009). Most of these studies used small number of subjects. Smell identification test has also been studied to differentiate patients with AD dementia from controls in various non-English population such as Chinese, Japanese, Norwegian, polish, Turkish (Chan et al., 2002, Suzuki et al., 2004, Kjelvik et al., 2007, Makowska et al., 2011).

A simple three-item test of olfactory identification differentiated AD patients (n=40) from those with major depression (n=20) (Solomon et al., 1998). Another study used a German odor identification test in 20 patients with AD, 20 with depressive disorder and 30 controls and found 100% sensitivity and 95% specificity with a score of 10/11 in differentiating AD from elderly with depression (Pentzek et al., 2007).

Smell identification test has also been studied as a marker for predicting conversion from mild cognitive impairment (MCI) to AD (Devanand et al., 2008, Devanand et al., 2000). This longitudinal study followed up 148 outpatients with MCI over 3 year period and found smell identification as one of the 5- predictors when combined strongly predicted conversion to due to AD. In a longitudinal study wherein annual clinical evaluations including smell identification test and brain autopsy at death was done for 471 older people without clinical manifestations of AD or MCI, and olfactory dysfunction was found to be related to both the level of AD pathology in the brain and the risk of subsequently developing prodromal symptoms of the disease, and these associations persisted after accounting for the effects of other recognized behavioral and genetic markers of the disease (Wilson et al., 2009).

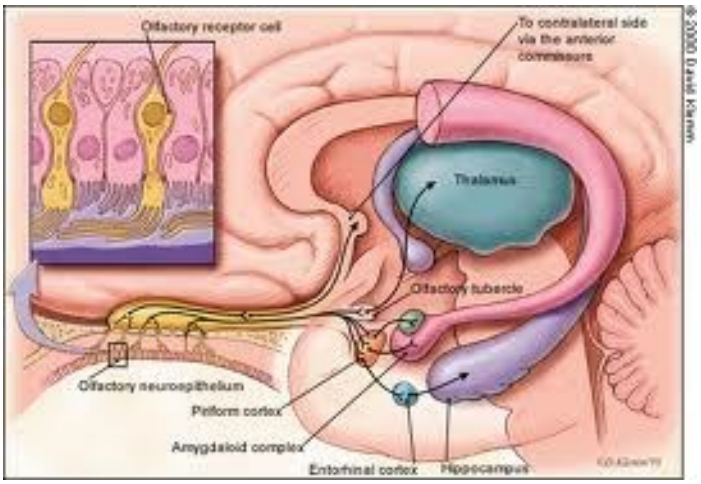
3.1.2 Anatomical correlates

The entorhinal cortex is a target of incoming olfactory information from the olfactory bulb, *via* the lateral olfactory tract. Entorhinal cortex supplies important input to the hippocampus CAI neurons, with feedback from the hippocampus to the mesial entorhinal cortex. In the case of olfactory input to the hippocampus, the entorhinal cortex represents the direct and substantial link between incoming olfactory input and the hippocampus. The entorhinal cortex is multimodal and its integrity is critical for flow of olfactory information. In addition to hippocampal projections, entorhinal cortex projects to orbital frontal cortex and receives input from frontal cortex and amygdala, areas important for olfactory function (Carmichael et al., 1994). Thus, the areas that evidence early neuropathy in AD, particularly in the mesial temporal lobe, are areas that, in addition to their well-known importance for memory function (Squire, 1992), are also critical for the processing of olfactory information.

The olfactory deficits in AD are also supported by missing electrophysiological response to olfactory stimulation (Peters et al., 2003). A strong relationship between left hippocampal volume losses in AD was found with performance on verbally based odour identification tasks (Murphy et al., 2003). It has been shown that involvement of the olfactory bulb and tract is one of the earliest events in the degenerative process on the central nervous system in AD (Christen-Zaech et al., 2003) and also that tau pathology in the olfactory bulb increases with severity of AD (Attems et al., 2005). A study showed that association with odour identification was robust for tangles in the entorhinal cortex and CA1/subiculum area of the hippocampus (Wilson et al., 2007a).

In summary, mesial temporal lobe, in addition to having importance for memory function is also critical for the processing of olfactory information (Figure 3.1).

Figure 3.1- Simplified diagram of cortical regions thought to be involved in the processing of olfactory information as it passes from the olfactory epithelium to the brain (Bromley, 2000)



3.1.3 Olfaction and cognition

Olfactory discrimination and identification have been more closely associated with higher cognitive functions and with subsequent cognitive decline (Sohrabi et al., 2009, de Wijk and Cain, 1994, Sohrabi et al., 2012). A strong association between cognitive functions and olfactory functioning has been reported and it has been concluded that compared with the ability to detect odours, identification of odours is more challenging, perhaps due to a lack of access to verbal or visual representations of odours (Richardson and Zucco, 1989). Similarly, Schab noted that odour identification may represent a semantic memory function (Schab, 1991). Some researchers suggest that olfactory identification is primarily predictive of memory decline (Swan and Carmelli, 2002).

Olfactory dysfunction has been suggested to be included in the diagnostic criteria of AD (Foster et al., 2008). Studies have reported association between olfactory impairment and subsequent cognitive decline in community dwelling older people (Wilson et al., 2007b, Wilson et al., 2006, Schubert et al., 2008, Sohrabi et al., 2012) and it has also been studied as a marker for predicting conversion from mild cognitive impairment to AD (Devanand et al., 2008, Devanand et al., 2000). Within AD patients, those who were carrying one or two ApoE epsilon4 alleles had a higher coefficient of correlation between the MMSE scores and the smell identification test scores than patients not carrying an ApoE epsilon4 allele (Suzuki et al., 2004).

However there is little known about association of olfactory identification impairments and cognitive decline with illness progression in AD dementia patients. In view of the common anatomical substrate for memory deficits and the olfactory function in AD, I hypothesized that olfactory identification ability at baseline would correlate with the cognitive ability and also predict altered cognitive function in a follow-up assessment. The specific questions examined by the current study were the following:

1) Is there any association between olfactory function and cognition in mild-mod AD dementia and can olfactory function predict future cognitive decline?
2) What is the difference between olfactory identification function between mild-mod AD dementia and non-demented control (NDC) subjects?
3) Is there any association between olfactory function and other non-cognitive symptoms in AD dementia?

3.2 Subjects and Methods

3.2.1 Subjects:

AD dementia : *Inclusion criteria*: Patients with probable mild to moderate dementia due to AD (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association [NINCDS-ADRDA] criteria) (McKhann et al., 1984), Mini Mental State Examination (MMSE) (Folstein et al., 1975) score range between 15 and 25, age 65 years or above. *Exclusion criteria*: significant neurological or psychiatric illness other than AD, significant unstable systematic illness or organ failure; medical conditions that may alter cerebral functioning or other conditions known to affect olfactory functioning (e.g., common cold, blocked nasal passages, polyps etc.). Subjects had either no history at all of cigarette smoking or had stopped smoking for 20 years or more. All were living in their own homes and had family members as reliable caregivers.

Non demented control (NDC): *Inclusion criteria*: MMSE score range between 24 and 30, CDR = 0, Geriatric Depression Scale score less than or equal to 5, age 65 years or above, medication stable, good general health. *Exclusion criteria*: Meet the DSM- IV criteria for dementia, significant neurological or psychiatric illness.

3.2.2 Recruitment methods:

AD dementia: Potential AD dementia participants were identified through discussion with the clinical team for mental health for older adults (MHOA) services of the South London and Maudsley (SLaM) NHS Foundation Trust and Mental Health Services for Older People (MHSOP), Leicestershire partnership NHS Trust.

Diagnosis of probable late onset dementia due to Alzheimer's disease was made according to NINCDS-ADRDA criteria (McKhann et al., 1984), following a semi-structured interview with the patient and an informant and detailed case history by the respective team in consultation with the team's senior clinician. All participants had investigations including neuroimaging as part of their routine clinical care and investigations.

NDC: Potential NDC were identified from other research projects at the department of Old Age Psychiatry, Institute of Psychiatry i.e., AddNeuroMed project, a European Union study, which has recruited a cohort of healthy elderly controls, AD and MCI subjects. (AddNeuromed:http://www.innomed-addneuromed.com).

Eligible participants were invited to participate in the study and given the information sheet. Informed consent was then sought from those who have indicated willingness to participate, after a "cooling-off period" of at least 24 hours. Especial care was taken to emphasise that refusal to participate, or withdraw from the study at any time, will not affect participant's clinical treatment in any way. Consent was also sought from the participants to obtain further demographic and clinical data from the medical notes. A time convenient for the participant was arranged for the assessment and was conducted at their own home or in outpatient clinic.

3.2.3 Study design and assessments

Baseline assessments were performed with a follow up assessment after three months.

The clinical assessments carried out were as described below:

3.2.3.1. *Mini-Mental State Examination* (MMSE) (Folstein et al., 1975)

General cognition was assessed using the MMSE which is a brief cognitive test used widely in clinical and research setting to screen for cognitive impairment and decline

(Salmon et al., 1990, Behl et al., 2005). The MMSE assesses orientation, immediate and short-term recall, attention, language and copying of a drawing. The MMSE has a maximum score of 30. It has been shown that the MMSE is equally sensitive to change in the mild to moderate demented Alzheimer patients (Salmon et al., 1990). A number of investigators have reported average annual rate of change (ARC) of approximately 2 to 4 points for the MMSE (Salmon et al., 1990, Behl et al., 2005, Galasko et al., 2000, Morris et al., 1993). The patients and families account of symptom progression was also noted in addition to loss of points on the MMSE in the previous 6 months.

3.2.3.2. Neuropsychiatric Inventory (NPI) (Cummings et al., 1994)

NPI is a useful instrument for characterizing the psychopathology of dementia syndromes and for assessing the efficacy of treatment. The NPI is based on a structured interview with a caregiver who is familiar with the patient. It evaluates 12 neuropsychiatric disturbances common in dementia: delusions, hallucinations, agitation, dysphoria, anxiety, apathy, irritability, euphoria, disinhibition, aberrant motor behaviour, night-time behaviour disturbances, and appetite and eating abnormalities. The severity and frequency of each neuropsychiatric symptom is rated and a total score calculated. Test-retest scores of all measures have been significantly correlated; with overall correlation of 0.79 for frequency ($p=0.0001$) and 0.86 for severity ($p=0.0001$) (Cummings, 1997).

3.2.3.3. Bristol Activities of Daily Living Scale (BADL) (Bucks et al., 1996)

The assessment is a carer rated instrument consisting of 20 daily-living abilities. It has good 'test-retest' reliability as measured by Cohen's Kappa and it correlates well with the Mini-Mental State Examination.

3.2.3.4. Olfactory identification test: University of Pennsylvania smell identification test (UPSIT) (Doty et al., 1984a)

UPSIT is a standardised test of odor identification with good test-retest reliability ($r=0.95$) and strong correlation with detailed olfactory threshold tests ($r=0.80$) (Doty et al., 1995). This "scratch 'n sniff" olfactory test consists of four booklets containing 10 odorants apiece; one odorant per page. The stimuli are embedded in 10-50microm

diameter microcapsules fixed in a proprietary binder and are positioned on brown strips at the bottom of each page. Above each odorant strip is a multiple-choice question with 4 response alternatives for each item (figure 3.2).

Only people fluent in English were recruited. Patients with history of language or speech difficulties (dysphasia) were not included. A formal testing for object recognition was not carried out.

Previous literature shows that one of the most dramatic ways to affect odor identification performance is to provide people with alternative labels for an odor they are trying to name. For familiar odors, identification performance is about 85% or better when alternative odor labels are provided (Cain and Krause, 1979, Doty et al., 1984b). This has been demonstrated and replicated in a study which demonstrated that recognition memory and odor naming were both better when the naming task provided participants with odor label alternatives (Frank et al., 2011).

UPSIT used in the current study provides 4 alternatives for each item, which are familiar odors from day to day life such as rose, onion, lemon etc. (Doty et al., 1984b).

Figure 3.2 - A volunteer being tested on the smell identification test

The items follow a standard format. An example is: 'this odour smells most like: a) chocolate; b) banana; c) onion; or d) fruit punch'. This is a forced choice procedure; for the test to be valid, participant must make a choice, even if they smell nothing. The tests were administered in a standard way used by the University of Pensylvania group, with one exception; the odor tapes were divided in half so that each booklet could be used for two subjects, done previously in AD studies (Warner et al., 1986). A short explanation was given at the first and then the odour was released by scratching the strip using the pencil provided with the booklet. The odour was immediately released from the test strip and the participant was given the booklet to smell. The participant smelled the strip on the booklet, and then read the four choices given. They were allowed to smell and read out as many times as they needed to make a choice. An examiner assisted all subjects with the test and allowed them unlimited time. The UPSIT has been used in British population to differentiate dementia patients from normal elderly British subjects tested in their homes (Gray et al., 2001) and as a treatment response marker (Velayudhan and Lovestone, 2009).

3.2.4 Statistical analysis:

Power Calculation: A total sample size of 46 will be required, assuming a conservative effect size (f^2) of 0.3, for UPSIT to predict MMSE decline, with a power of 0 .95 and alpha error probability of 0.05 (2-tailed) in a linear regression model (Faul et al., 2009).

Data analysis: SPSS 20.0, STATA 10 and Excel 2010 were used for statistical analysis of the data. Chi-Square, student's t-test, correlation analysis (Spearman non-parametric test) and non-parametric Mann-Whitney tests was used for comparing the socio-demographic and clinical parameters (MMSE, NPI, UPSIT and BADL) between groups: AD dementia subjects and NDC; rapid cognitive decliners and non-rapid cognitive decliners. Linear regression was performed with the MMSE scores over follow up as the dependent variable and baseline UPSIT scores, age, baseline MMSE scores, duration of illness, gender, education and follow up time as predictive variables within the whole Alzheimer's disease sample.

Patients were classified as AD dementia Cognitive Rapid Decliners (RCD), based on a decline of ≥2 points at baseline in the previous 6 months, of Mini Mental State

Examination (MMSE), a commonly used cognitive test to assess severity and decline in clinical settings (Behl et al., 2005) .

Using a median split, AD dementia participants were divided into two groups: those with more olfactory dysfunction i.e., olfactory rapid progressors (UPSIT≤15; *n* = 25, mean UPSIT = 13.4 [4.3]) and those with lesser impaired olfaction (UPSIT ≥16; *n* = 32, mean UPSIT = 19.6 [4.1]). Comparisons were made on demographic information, clinical characteristics, and cognitive and behavioural test results with parametric (students *t*-tests) and nonparametric (Chi-square test, Mann-Whitney tests) statistics, as appropriate. Alpha level was set at 0.05; corrections for multiple comparisons were not made given the exploratory nature of the study. The demographic data, clinical characteristics, and cognitive performance of the patients in each group are presented in Table 3.3.

Further a linear mixed effects model using STATA 10 was fitted to investigate whether baseline UPSIT score was associated with overall MMSE levels measured in the two visits. The average baseline MMSE and the average change in the MMSE over follow-up time was calculated for all subjects as a group (fixed effects) and subject-specific intercept and slope terms which reflected deviation from the group average (mixed linear effects) were calculated. The calculation included adjustment for follow-up time, age, disease duration, gender and cholinesterase inhibitor treatment. A significant interaction between baseline UPSIT score and follow- up time (Visit) was used to test the null hypothesis that there was no difference in the rate of cognitive decline i.e. in slopes for different baseline UPSIT scores.

Informed consent or assent as appropriate was taken from all the participants. Ethical approval was obtained from local ethic committees (Joint SLaM & Institute of Psychiatry Ethics ref no. 05/Q0706/281 and SSA approval from LPT- ELMH0555/ 2011).

3.3 Results

Of 64 AD dementia patients who were eligible and agreed to participate in the study, 57 successfully completed both the baseline and follow up assessments. 7 patients dropped out owing to developing stroke, being hospitalised or moving to institutional care in a different locality. In the NDC group, out of the 28 subjects recruited, 25 subjects completed both the baseline and follow up assessments.

Among the AD dementia participants some received either donepezil 5 mg/day (n=18) or galantamine 8mg/day (n= 13) for initial 4 weeks, which was then increased to 10mg/day (donepezil) or 24 mg/day (galantamine) by their respective clinical teams as per the local Trust policy respectively. 3 patients were already on donepezil 10 mg/day at the point of recruitment. Some patients (n=23) were not on cholinesterase inhibitors because of reasons such as cardiac contraindications, intolerance, patients not willing for therapy or compliance issues. None of the patients reported subjective impairment in olfaction. All participants were white Europeans, except for one male and a female in each group.

Within the AD dementia subjects, 28 were classified as rapid decliners as defined by the on-priori definition with loss of 2 or more MMSE scores in the previous 6 months.

3.3.1 Association of olfactory function with baseline cognition and prospective cognitive decline in mild-mod AD dementia

As described above, the AD dementia subjects were classified as RCD (n=28) and Non-Rapid Cognitive Decliners (NRCD) (n=29), based on a decline of ≥2 MMSE points in the previous 6 months, at the baseline. The 2 groups were not different in age, gender, duration of illness; follow up period, family history or the number of subjects on cholinesterase inhibitors therapy. However, the RCD had lower baseline MMSE (statistically significant) and UPSIT (statistically not significant) measures compared to NRCD (Mann-Whitney U test) (table 3.1b, figure 3.3). The follow up MMSE and UPSIT scores were both low for the RCD compared to NRCD (statistically significant) (Mann-Whitney U test) (table 3.1b, figure 3.3). The baseline UPSIT score correlated with both

baseline and follow up MMSE ($p<0.01$, $r^2=0.4$) and follow up UPSIT scores ($p<0.001$, $r^2=0.6$).

Linear regression analysis showed baseline UPSIT scores, baseline MMSE scores and gender as a better predictor factor for follow up MMSE scores, in unadjusted models than variables such as age, gender, duration of illness, education and follow-up time (Table 3.3). However in the adjusted model baseline UPSIT scores was no more significant (Table 3.3).

Linear mixed effects model adjusted for follow-up time, age, disease duration, gender and cholinesterase inhibitor treatment showed that although baseline UPSIT was associated with lower MMSE at the baseline, the interaction between baseline USPIT and time was not significant ($p= 0.201$), indicating no association between baseline UPSIT and MMSE change (decline) over the two time points.

Table 3.1- Comparison of socio-demographic-clinical parameters between the groups: a) AD dementia subjects and non-demented controls; b) Within AD dementia cohort: rapid cognitive decliners and non-rapid cognitive decliners

a. AD dementia subjects and non-demented controls				b. Comparisons within AD dementia subjects (n=57)		
Variables	**AD dementia (n=57)**	**NDC (n=24)**	**p-value**	**Cognitive rapid decliners (n=28)**	**Cognitive slow decliners (n=29)**	**p-value**
Female/male	35/22	14/10	N.S[a]	16/12	19/10	N.S[a]
Age, years	81.4 (5.4)	77.3 (6.6)	<0.01*	80.6 (5.7)	82.1 (5.1)	N.S*
Education	10.6 (1.4)	14.2(4.7)	<0.001*	10.7 (1.5)	10.4(1.3)	N.S*
Duration of illness	27.8 (27.1)	n/a	n/a	27.5 (21.5)	28.1(22.4)	N.S*
MMSE baseline	21.6 (3.7)	29.1(0.9)	<0.001*	20.1 (2.9)	23.0(3.5)	0.001*
MMSE FU	21.7 (3.9)	29.2 (0.7)	<0.001*	20.4 (3.5)	22.9 (4.0)	0.02*
UPSIT baseline	16.1(5.3)	28.6 (5.8)	<0.001*	15.0 (4.9)	17.2 (5.2)	0.07*
UPSIT FU	16.1 (5.4)	28.8 (5.9)	<0.001*	14.4 (5.3)	17.8 (5.2)	0.02*
BADL baseline	9.3 (7.3)	n/a	n/a	9.5 (7.6)	9.1 (7.1)	N.S*
BADL FU	9.6 (7.1)	n/a	n/a	9.4 (7.6)	9.9 (6.6)	N.S*
NPI baseline	7.0 (9.9)	n/a	n/a	7.1 (9.2)	6.9 (10.8)	N.S*
NPI FU	5.5 (7.9)	n/a	n/a	5.2 (5.6)	5.7 (9.5)	N.S*
Family H	16 (34%)	0%	0.001[a]	8 (28.6%)	8 (27.6%)	N.S[a]
Follow-up in weeks	19.9 (10.1)	28.1(11.9)	<0.01*	21.8 (13.2)	18.2 (5.4)	N.S*
UPSIT time	26.7(9.2)	18.4 (6.4)	<0.01*	26.9 (10.6)	26.6 (7.6)	N.S*
CI Therapy	34 (59.6%)	n/a	n/a	14 (50%)	20 (69%)	N.S[a]

Values are mean (SD) or n (%); [a] Calculated using the χ^2 test, [b] Calculated using the t test, *Wilcoxon paired test AD, Alzheimer's disease; NDC, non-demented controls; MMSE, Mini Mental State Examination; UPSIT, University of Pennsylvania Smell Identification Test; CI, Cholinesterase inhibitor; FU, follow up; BADL, Bristol Activities of daily living, NPI, Neuropsychiatric Inventory; n/a, not applicable

Table 3.2 - Linear regression analysis with the follow up MMSE scores as the dependent variable and baseline UPSIT scores, age, baseline MMSE scores, duration of illness, gender, follow up in weeks and cholinesterase therapy alternatively (Model 1) or simultaneously (Model 2) entered as predictive variables within the whole Alzheimer's disease sample.

	R^2 (%)	Beta	T-value	P value
Model 1				
UPSIT baseline	0.16	0.401	3.243	<0.01*
Age in years	0.01	0.075	0.560	0.578
Education	0.00	-0.017	-0.122	0.903
Duration of illness	0.03	0.179	1.337	0.187
MMSE baseline	0.49	0.698	7.237	<0.001*
Gender	0.08	-0.285	-2.209	0.031*
Follow up in weeks	0.01	-0.112	0.837	0.406
CI therapy	0.01	0.085	0.631	0.531
Model 2				
UPSIT baseline		0.165	1.685	0.098
MMSE baseline		0.619	6.321	<0.001*
Gender		-0.205	-0.229	0.030*

R^2 (%) = R^2 value in percent for the overall model; *, $p < 0.05$
MMSE, Mini Mental State Examination; UPSIT, University of Pennsylvania Smell Identification Test; CI, Cholinesterase inhibitor

3.3.2 Comparison of AD dementia and NDC subjects:

The socio-demographic and clinical comparison between the AD dementia subjects and the NDC are as described in Table 3.1a. The NDC were younger and had higher education. They scored higher on the baseline MMSE and UPSIT measures. The AD dementia patients took longer to complete the UPSIT and more AD dementia patients had a family history of dementia. At follow up the MMSE and the UPSIT loss was not different from baseline within the groups (table 3.1a, figure 3.3). The subjects who dropped out were similar to the subjects in main data in their demographic and baseline data. The 7 AD dementia patients had mean age of 84.6 years (±5.5), 12.8 years of education (±2.7), mean MMSE scores 21 (±4) and mean UPSIT scores 14.4 (±7.3). The 4 NDC subjects had mean age of 77.5 years (±11.5), 15 years of education, mean MMSE scores 28.8 (±0.5) and mean UPSIT scores 26.5 (±11.8).

Figure 3.3 - Line plot representation of baseline and follow up MMSE and UPSIT scores between subjects with Alzheimer's disease dementia (Rapid Cognitive Decliners and Non Rapid Cognitive Decliners) and non-demented controls

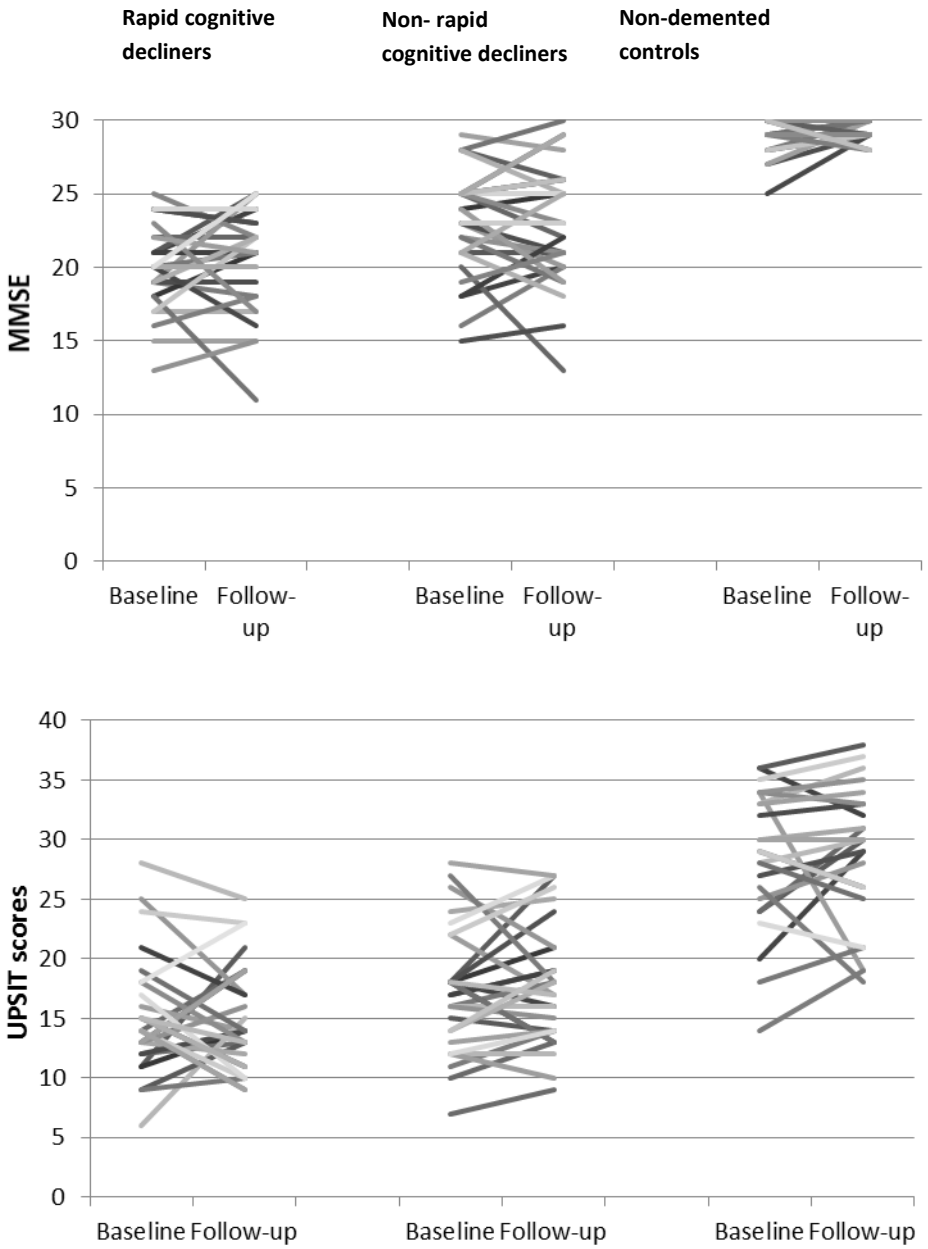


Table 3.3 - Characteristics and cognitive performance of rapid olfactory progressors and slow olfactory progressors AD patients

Variables	Rapid Olfactory Progressors (n=25)	Slow Olfactory Progressors (n=32)	p-value
Female/male	17/8	18/14	N.S[a]
Age, years	81.9 (6.0)	80.9 (4.9)	N.S*
Education	10.8 (1.5)	10.3(1.2)	N.S*
Duration of illness	27.3 (19.0)	28.1 (24.0)	N.S*
MMSE baseline	20.2 (3.1)	22.7(3.6)	0.01*
MMSE FU	19.7 (3.1)	23.3 (3.3)	0.001*
UPSIT baseline	11.8(2.2)	19.6 (4.1)	<0.001*
UPSIT FU	13.4 (4.5)	18.3 (5.2)	0.001*
BADL baseline	13.5 (7.3)	6.0 (5.4)	<0.001*
BADL FU	12.5 (8.1)	7.0 (4.8)	<0.01*
NPI baseline	9.7 (11.2)	5.0 (8.6)	0.05*
NPI FU	7.4 (5.7)	4.2 (9.2)	<0.01*
Family H	7 (28%)	9 (28%)	N.S[a]
Follow-up in weeks	20.6 (11.3)	19.4 (9.3)	N.S*
UPSIT time	27.4(11.5)	26.2 (6.9)	N.S*
CIs	16 (64%)	18 (56%)	N.S[a]

Values are mean (SD) or n (%); [a] Calculated using the χ^2 test, [b] Calculated using the t test, *

Wilcoxon paired test; AD, Alzheimer's disease; NDC, non-demented controls; MMSE, Mini Mental State Examination; UPSIT, University of Pennsylvania Smell Identification Test; CI, Cholinesterase inhibitor; FU, follow up; BADL, Bristol Activities of daily living, NPI, Neuropsychiatric Inventory

3.3.3 Association between olfactory function and other non-cognitive symptoms in AD dementia

Using a median divide the AD dementia participants were further classified into Rapid Olfactory Progressors, ROP, (UPSIT≤15; *n*=25) and Slow Olfactory Progressors, SOP, (UPSIT ≥16; *n*= 32). The two groups were similar in their socio-demographic data, family history, follow-up time and cholinesterase inhibitors treatment (table 3.3). However the ROP subjects had lower MMSE and lower UPSIT implying more cognitive and olfactory impairment (table 3.3). They also had higher BADL and NPI scores reflecting more functional deficits and more non-cognitive behavioural and psychological symptoms (table 3.3).

3.4 DISCUSSION

Olfactory dysfunction has been studied as a diagnostic marker as well as a marker of conversion in AD dementia . However, this is the first study to investigate olfactory dysfunction as a severity and progression marker in dementia due to AD to my knowledge. AD dementia participants were deliberately chosen in the early stages (mild-moderate) of the disease, so that there is little question of their ability to understand and perform the smell test. None of the AD dementia participants had speech or language difficulties.

3.4.1 Olfaction in subjects with AD dementia and non-demented control (NDC):

AD dementia patients had lower olfactory identification scores compared to NDC, which has been reported previously in a number of studies (Warner et al., 1986, Serby et al., 1991, Richardson and Zucco, 1989, Doty et al., 1987, Larsson et al., 1999, Makowska et al., 2011, Bahar-Fuchs et al., 2011, Tabert et al., 2005). The median UPSIT score for the AD dementia subjects in the study was 15, similar to previous reports from British population (Gray et al., 2001, Velayudhan and Lovestone, 2009b). The UPSIT scores of the NDC subjects in the cohort too were in keeping with previous studies in a similar age group with mean UPSIT scores of 31.8 (±7.2) and mean age 70.1yrs (±5.3) (Serby et al., 1991). As a group there was no difference in the olfactory performance between the genders as previously reported (Westervelt et al., 2007).

3.4.2 Olfaction and cognition:

This study showed clear evidence of a relation of olfactory function with cognition. A) Olfactory identification function was correlated with baseline cognition. B) The rapid and slow olfactory progressors correlated with the rapid and non-rapid cognitive decliners group.

Previous studies have found strong correlations between olfactory identification and cognitive performances (Serby et al., 1991, Larsson et al., 1999, Hidalgo et al., 2011). Olfactory discrimination and identification have been more closely associated with higher cognitive functions and subsequent cognitive decline (Sohrabi et al., 2009, de Wijk and Cain, 1994, Sohrabi et al., 2012). A large-scale study in older adults (n=1920) on the relationship between olfactory identification ability and general cognitive functioning (as measured by MMSE) indicated that olfactory dysfunction at baseline was significantly predictive of future cognitive impairment after 5 years (odds ratio (OR)=6.62; confidence interval (CI)=4.36–10.04) (Schubert et al., 2008). A strong association between cognitive functions and olfactory functioning has been reported and it has been concluded that compared with the ability to detect odours, identification of odours is more challenging, perhaps due to a lack of access to verbal or visual representations of odours (Richardson and Zucco, 1989). Similarly, Schab noted that odours identification may represent a semantic memory function (Schab, 1991). Some researchers suggest that olfactory identification is primarily predictive of memory decline (Swan and Carmelli, 2002). In a functional magnetic resonance imaging (fMRI) study, the blood oxygen level-dependent (BOLD) signal at primary olfactory cortex (POC) was found to be weaker in AD than in healthy control subjects (Wang et al., 2010). At the lowest odorant concentration, the BOLD signals within POC, hippocampus, and insula significantly correlated with UPSIT, MMSE, DRS-2, and CDR scores, demonstrating that olfactory fMRI is sensitive to the AD-related olfactory and cognitive functional decline (Wang et al., 2010).

3.4.3 Olfaction and non-cognitive symptoms:

The present study showed that higher olfactory impairment was associated with more dependence in functional abilities at the baseline. Olfactory function has been

associated with functional dependence previously in subjects with MCI and normal elderly (Wilson et al., 2007b). A previous report from our cohort had shown that olfaction scores predicted improvement better than cognitive scores as indicated by global and functional improvement in AD dementia patients receiving cholinesterase inhibitor therapy (Velayudhan and Lovestone, 2009).

Interestingly the present study also found an association in the olfactory rapid decliners group with behavioural symptoms (NPI) (Table3.3). This has not been reported previously. This could be as most of the previous studies have focussed association of olfaction with cognition. This needs to be explored further in future studies.

On the whole, the study results reflect that higher olfactory impairment is indicative of more symptomatic illness or severity, i.e., lower cognition, higher functional dependence and presence of behavioural symptoms.

The baseline UPSIT scores predicted the follow up MMSE in an unadjusted model, however, losing this effect in adjusted model with MMSE and gender. Further a linear mixed effect model showed that although baseline UPSIT was associated with MMSE at the baseline, there was no association between baseline UPSIT and MMSE change (decline) over the two time points. This could have been the influenced by the cholinesterase inhibitors therapy in some patients which influences UPSIT scores more than the cognitive scores (Velayudhan and Lovestone, 2009a) and also the short follow up period. Also there were more females than males in the cohort, who perform better on the olfactory tasks than men (Murphy et al., 2002, Mullol et al., 2012).

Main limitations of the study are its small sample size and single point follow-up over a short duration. Longer follow up with multiple point testing and assessments of cognitive, functional and behaviour changes, would be more informative of predictive ability of the olfactory function for illness progression in AD dementia.

Gender was predictive of lower follow-up MMSE scores, with women losing more MMSE points over follow up period. The possible explanation could be that women have a higher risk of developing AD above 80 years of age (Copeland et al., 1999). The present

AD dementia cohort had a mean age above 80 years (81.5), so the progression too must have been faster in women than men.

A diminished sense of smell has practical implications in relation to AD dementia, such as decreased appetite, with resultant weight loss and poor nutritional status. Other problems may be the inability to detect noxious odours such as gas and smoke. Patient and family, both should be made aware of this deficit and the potential problems this may cause.

In conclusions, the study confirms associations of olfaction with cognition in mild to moderate AD dementia and supports the utility of the smell identification function as an adjunct clinical measure to assess severity in AD dementia. Further work, including larger longitudinal studies, is needed to explore its value in predicting AD dementia progression.

3.4 **My role:**

Following detailed literature review; I generated the hypothesis to be tested; established the aims of the study; and conceptualised and conceived the study. After obtaining the ethic approval I recruited the subjects and completed the assessments; carried out statistical analysis; interpretation of the results; and wrote the chapter. My supervisors Prof Simon Lovestone and Dr John Powell provided overall guidance.

Chapter 4

PLASMA PROTEINS AS MARKERS OF PROGRESSION IN ALZHEIMER'S DISEASE

4.1 Paper 1

Association of Plasma Clusterin Concentration with Severity, Pathology, and Progression in Alzheimer Disease

Thambisetty M, Simmons A, Velayudhan L, et al. Archives of General Psychiatry, 2010

4.1.1 MY CONTRIBUTION

In this novel proteomic-neuroimaging discovery paradigm; a multi-authored complex paper which used multiple techniques, I made an essential contribution towards the discovery and identification of the clusterin, in human plasma, as a marker of disease progression in Alzheimer's disease (AD) dementia.

'Slow vs fast progressors in AD' section of the paper

In this study I contributed to the design, carried out the proteomic experiments, did detailed statistical analysis, and interpretation as described below. I contributed to writing up of this part of the manuscript and to the necessary corrections following submission and anonymous peer review process, under the supervision of Prof Simon Lovestone who was the principal investigator of this collaborative project and the corresponding author.

The methods and results for identification of the plasma protein clusterin, as AD progression marker, are described below:

4.1.2 METHODS

4.1.2.1 Samples and subjects

Samples used came from two studies – the Alzheimer's Research Trust funded cohort at KCL (KCL ART) (Hye et al., 2006) and AddNeuroMed (Lovestone et al., 2007a) studies. The KCL ART study is a cohort of people with AD, MCI and normal elderly started in 2001 for the purpose of biomarker discovery and validation. All subjects were white UK citizens with grandparents born in the UK and are assessed annually. AddNeuroMed is a cross-European cohort founded for biomarker discovery; AD dementia cases are assessed 3 monthly in the first year and annually thereafter, MCI and control groups are

assessed annually. All subjects are white Europeans recruited from UK, France, Italy, Finland, Poland and Greece. Cases with probable AD according to NINCDS-ADRDA criteria were recruited through secondary care as previously described (Hye et al., 2006) and evaluated with a standardised assessment previously shown to have high diagnostic validity against assessment at post mortem (Foy et al., 2007)(Institute of Psychiatry ethics number 06/Q0706/50). The full standardized assessment includes demographic and medical information, cognitive assessment including MMSE (both studies), ADAS-cog (AddNeuroMed only) and CERAD battery, and scales to assess function, behavior and global levels of severity including the CDR. Cases with amnestic MCI were defined as subjective memory complaint, CDR score <1 and evidence for objective memory impairment using the CERAD delayed word list recall (-1.5SD cut off). MCI cases were recruited from both primary and secondary care. Normal elderly controls, defined as having no evidence of cognitive impairment, were recruited systematically from primary care patient lists in the case of the KCL ART study and from primary care and from elsewhere in the AddNeuroMed study. Peripheral venous blood has been collected at baseline (initial assessment) and at subsequent time points and stored at -80°C according to rigorous standard procedures. The blood samples are collected in 9ml EDTA tube for plasma.

4.1.2.2. Discovery-phase proteomic experiments

4.1.2.2.1 Subjects and samples

I examined samples from 51 subjects with mild-moderate AD dementia (NINCDS-ADRDA criteria; target MMSE>10) from the AddNeuroMed cohort. Patients were characterised as fast progressors based on a decline of 2 or more points on the Alzheimer disease assessment scale – Cognitive (ADAS-COG) score from baseline to the 6-month follow-up time point. Using this criterion, I characterised 22 subjects as 'fast' and 29 as 'slow' progressors. The two groups were well matched by age, gender and baseline ADAS-COG and MMSE scores. All subjects in both groups were on acetylcholinesterase inhibitor treatment. Plasma samples used for the proteomic experiments were obtained at the baseline evaluation.

4.1.2.2.2 2D Polyacrylamide Gel Electrophoresis (2-D PAGE)

I analysed the plasma samples from selected AD dementia subjects from the AddNeuroMed cohort using 2DGE and followed it by tandem mass spectrometry as previously described (Hye et al., 2006). Briefly in 2DGE plasma proteins were separated in the first-dimension according to isoelectric points, on immobilised pH (3-11) gradient strips. This was followed by the second-dimension step, SDS polyacrylamide gel electrophoresis, separating proteins according to molecular weight. Separated proteins were then visualised following silver staining.

I used Progenesis SameSpots v3.0 (Nonlinear Dynamics) for 2D gel image analysis and to obtain the spot normalised values from each gel. Prominent spots were used to manually assign vectors to each gel image. The vectors were used to warp the images and align the spot positions to a common reference gel. Image warping is a nonlinear deformation, basically a smooth mapping between 2 image planes that maps every point in one image to a point in another; in this case every spot in an image to a point in the reference image.

The proprietary warping algorithms used by Nonlinear Dynamics spatially align common motifs within different gel images to a common reference gel, thus compensating for any distortions of protein spot patterns arising from gel-to-gel variation. Nonlinear Dynamics uses a combination of image warping and matching to achieve the best possible matching data, to compare identical protein spots across different gels, facilitating the exploration of expression changes under different experimental conditions.

Spot detection was performed on this reference gel after editing and removing artefacts after visual checking of each spot. Of the 1157 spots identified, I successfully matched 413 spots across every gel between the two subject groups.

Statistics

Statistical analysis was performed using SPSS (15.0) and Partial least squares discriminant analysis (PLS-DA). Shapiro-Wilk test was done to test for normality of the integrated optical densities (OD) of spots on the 2D gels. Multivariate analysis (PLS) was

done to derive a panel of protein spots that discriminated between fast and slow progressor groups of AD dementia patients. Non-parametric tests (Mann-Whitney) with correction for multiple comparison tests was then used to compare individual spot OD between subject groups. I found 27 spots that were significantly different between the 2 groups with fold changes between 1.1-1.4. Based on protein spots with highest Variable of Importance (VIP), 9 spots were excised, proteolysed and the resulting peptides analysed using LC/MS/MS (figure 4.1).

4.1.2.2.3 Mass spectrometry

Protein spots of interest were excised washed and in-gel digested with trypsin. Peptides were extracted by acetonitrile and aqueous washes and analysed by LC/MS/MS as previously reported (Hye et al., 2006). The mass spectral data were processed into peptide peak lists and searched against the Swiss-Prot Database using Mascot software (Matrix Science, UK). LC/MS/MS analysis successfully determined protein identifications from each of the 2D gel spots provided which included Clusterin and transthyretin (table 4.1), subsequently validated and published (Thambisetty et al., 2010, Velayudhan et al., 2012).

Figure 4.1 - A representative of 2 dimensional gel electrophoresis blot of from one of the AD plasma sample with spots of interest outlined in blue

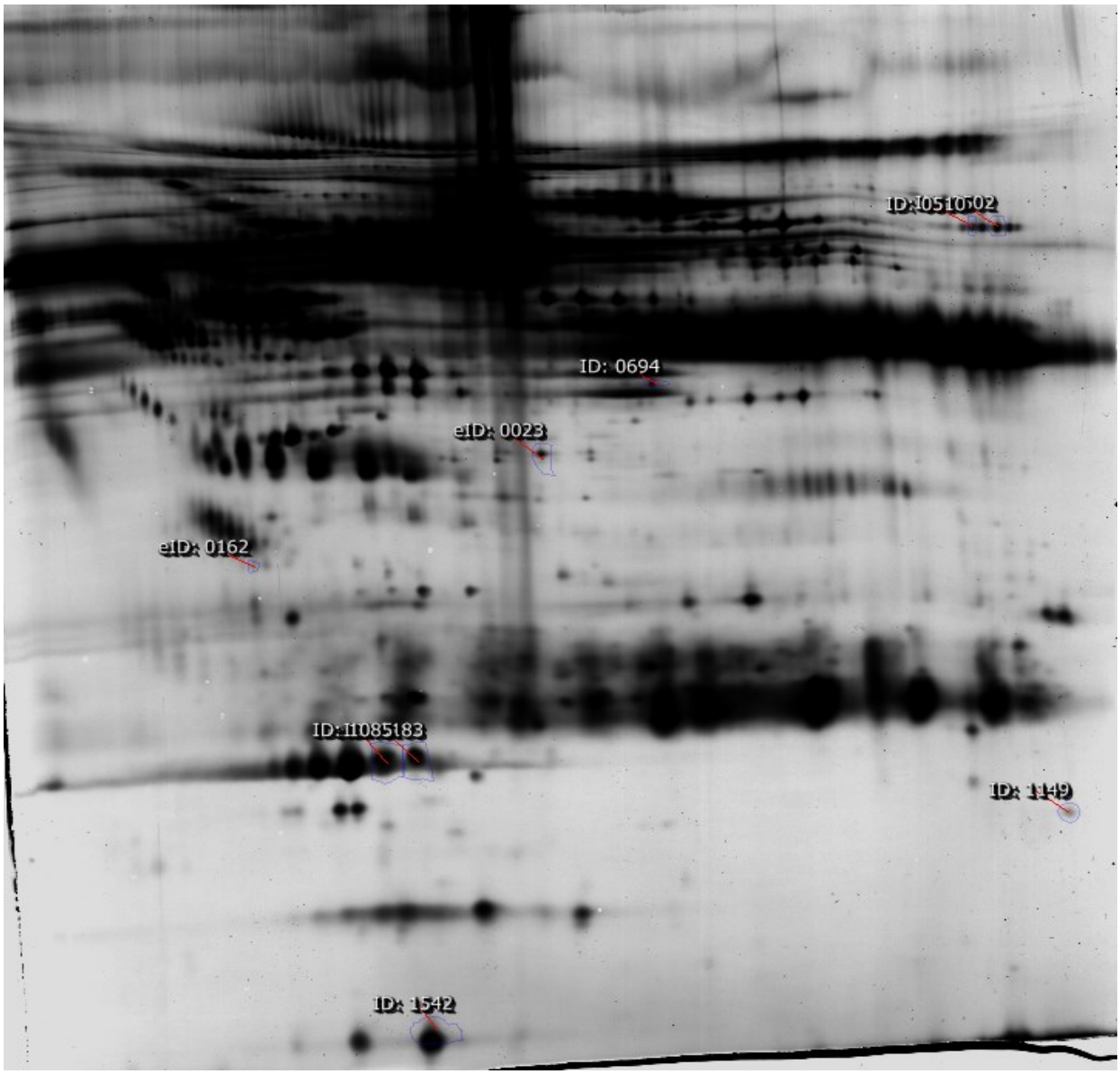


Table 4.1 - Results of LC/MS/MS analysis on nine 2D gel spots of interest of Human plasma

Spot ID	Protein I.D.	Species	Accession No.	MW (Da)	pI	No. Peptides Matched	Percentage Coverage	Sequence Matched
0023	Complement C4-A precursor	Human	P0C0L4	192650	6.65	7	6%	
694	Fibrinogen gamma chain precursor	Human	P02679	51479	5.37	11	38%	
	Hemopexin precursor	Human	P02790	51643	6.55	7	18%	
	Ig gamma-4 chain C region	Human	P01861	35918	7.18	3	11%	GPSVFPLAPCSR STSESTAALGCLVK NQVSLTCLVK
1149	Complement component C8 gamma chain precursor	Human	P07360	22264	8.49	8	54%	
162	Clusterin precursor	Human	P10909	52461	5.89	5	10%	EIQNAVNGVK TLLSNLEEAK KTLLSNLEEAK ASSIIDELFQDR EPQDTYHYLPFSLPHR
510	Complement C4-A precursor	Human	P0C0L4	192650	6.65	23	16%	
	Ig gamma-1 chain C region	Human	P01857	36083	8.46	5	22%	GPSVFPLAPSSK FNWYVDGVEVHNAK NQVSLTCLVK GFYPSDIAVEWESNGQPENNYK TTPPVLDSDGSFFLYSK
502	Complement C4-A precursor	Human	P0C0L4	192650	6.65	26	15%	
	Ig gamma-3 chain C region	Human	P01860	32310	7.89	2	9%	SCDTPPPCPR EPQVYTLPPSREEMTK
	Ig gamma-1 chain C region	Human	P01857	36083	8.46	2	7%	FNWYVDGVEVHNAK NQVSLTCLVK
183	Apolipoprotein A-I precursor	Human	P02647	30759	5.56	34	86%	
1085	Apolipoprotein A-I precursor	Human	P02647	30759	5.56	36	84%	
	Apolipoprotein M	Human	O95445	21239	5.66	6	26%	
	Lysozyme C precursor	Human	P61626	16526	9.38	1	8%	STDYGIFQINSR
	Serum albumin precursor	Human	P02768	69321	5.92	1	1%	LVNEVTEFAK
1542	Transthyretin precursor	Human	P02766	15877	5.52	13	73%	

4.1.2.3 Validation-phase experiments

4.1.2.3.1 Clusterin

Clusterin was a protein found common in the discovery study and another study by a colleague within the centre, comparing AD dementia patient and controls. Therefore, further validation of clusterin protein was planned, in larger cohort, to test for its robustness as a progression marker.

Clusterin, a major glycoprotein was identified by Irving Fritz and his laboratory in 1983 and named so since it was able to aggregate several cell types, e.g. Sertoli cells, and was involved in spermatogenesis (Fritz et al., 1983). Clusterin has been called a number of different names due to its versatile functional capacities, e.g. apolipoprotein J (ApoJ), sulfated glycoprotein-2 (SGP-2), secreted glycoprotein gp80, complement-associated protein SP-40,40, complement lysis inhibitor (CLI), and testosterone repressed prostate message 2 (TRPM-2).
Clusterin was approved as the official name at the First International Clusterin Workshop in 1992.

The first time that clusterin was associated to Alzheimer's disease was in work done in the laboratory of Caleb Finch (May et al., 1990). They demonstrated that the expression of clusterin was clearly increased in hippocampal samples of patients with AD compared to the age matched controls. After these original observations, the role of clusterin has been extensively studied in the pathogenesis of AD (Nuutinen et al., 2009a). It was observed that clusterin can bind amyloid-β peptides and prevent their fibrillization (Ghiso et al., 1993, Zenkel et al., 2006). Clusterin is also involved in the clearance of amyloid-β peptides and fibrils by binding to megalin receptors and enhancing their endocytosis within glial cells (Hammad et al., 1997, Cole and Ard, 2000, Bartl et al., 2001, Wang et al., 2006). Clusterin is a complement inhibitor and can suppress complement activation observed in AD (Choi-Miura et al., 1993, Zwain et al., 1994, Santilli et al., 2003). Clusterin is also present in lipoprotein particles and regulates cholesterol and lipid metabolism of brain which is disturbed in AD (Calero et al., 1999, Reid et al., 2007, Hooijmans and Kiliaan, 2008, Hirsch-Reinshagen et al., 2009). Clusterin is a stress-induced chaperone which is normally secreted, but in conditions of cellular

stress, it can be transported to cytoplasm where it can bind to Bax protein and inhibit neuronal apoptosis (Zhang et al., 2005). Clusterin can also bind to Smad2/3 proteins and potentiate the neuroprotective transforming growth factor- β (TGFβ) signalling (Lee et al., 2008). An alternative splicing can produce a variant isoform of clusterin which can be translocated to nuclei where it induces apoptosis (Leskov et al., 2003).

To confirm the findings from the discovery phase that the plasma clusterin levels was altered between fast and slow progressors, quantitative analysis of clusterin was carried out in a larger independent cohort of AD dementia patients and correlated with the rate of cognitive decline and severity.

4.1.2.3.2. ***Subjects and samples details***

Samples used came from two studies – the Alzheimer's Research Trust funded cohort at KCL (KCL ART) (n=114) (Hye et al., 2006) and AddNeuroMed (n=239) (Lovestone et al., 2007a) studies. The KCL ART study is a cohort of people with AD, MCI and normal elderly started in 2001 for the purpose of biomarker discovery and validation. All subjects were white UK citizens with grandparents born in the UK and assessed annually. AddNeuroMed is a cross-European cohort founded for biomarker discovery; AD cases were assessed 3 monthly in the first year and annually thereafter, MCI and control groups assessed annually. All subjects were white Europeans recruited from UK, France, Italy, Finland, Poland and Greece. Cases with probable AD according to NINCDS-ADRDA criteria were recruited through secondary care as previously described (Hye et al., 2006) and evaluated with a standardised assessment previously shown to have high diagnostic validity against assessment at post mortem (Foy et al., 2007). The full standardized assessment includes demographic and medical information, cognitive assessment including MMSE (both studies), ADAS-cog (AddNeuroMed only), and scales to assess function, behaviour and global levels of severity including the CDR. Normal elderly controls, defined as having no evidence of cognitive impairment, were recruited systematically from primary care patient lists in the case of the KCL ART study and from primary care and from elsewhere in the AddNeuroMed study.

Peripheral venous blood has been collected at baseline (initial assessment) and at subsequent time points and stored at -80°C according to rigorous standard procedures.

The blood samples were collected in 9ml EDTA tube for plasma. All samples were collected in the morning following a 2 hour fasting. They were processed and stored within 2 hours of collection.

Plasma samples from AD dementia patients identified from both the AddNeuroMed (n=239) and KCL-ART (n=114) with longitudinal assessments over 1 year studies were used for these experiments. Since ADAS-Cog scores were not obtained in the KCL-ART study, rate of decline in MMSE scores was used for classification of fast and slow progressors. MMSE scores obtained at the baseline blood sampling were used to derive an annualised retrospective progression rate in order to stratify AD patients into fast and slow progressors by using the equation: Progression rate = (30)-(MMSE score at the time of blood sampling)/duration of illness in years. The fast progressors were those with a decline of more than 2 MMSE points per year. Similarly, an annualised prospective progression rate was calculated in the combined AddNeuroMed and KCL-ART cohorts by calculating the decline in MMSE score one year after blood sampling and those with a decline of more than 2 MMSE points per year were defined fast progressors.

4.1.2.3.3 Clusterin ELISA assay

Plasma concentration of clusterin was assayed by a commercially available ELISA kit (Human Clusterin ELISA, RD194034200R, Biovendor Laboratory Medicine Inc). All samples were run in duplicate and the overall coefficient of variance (CV) was 3.5%.

4.1.2.3.4 Statistics

Inter-group differences in age and education were tested by univariate general linear models. Differences in plasma clusterin concentration between fast and slow progressing AD dementia patients were tested by univariate general linear models after covarying for duration of disease in the analysis for retrospective rate of decline. As there was no significant difference in duration of illness for the two groups of prospective decliners, concentration in these patients was examined by an independent samples t-test.

4.1.3 RESULTS:

The sociodemographic and clinical parameters for rapid and non-rapid progressors in the discovery and validation phase are demonstrated in table 4.2 and 4.3. A significant increase in clusterin concentration in AD dementia patients was observed with accelerated cognitive decline prior to blood sampling (Figure-4.2) (ANCOVA; n=344; t(341)=3.40; p=0.0007; duration of disease as covariate) and an increase in clusterin concentration in AD dementia patients with faster cognitive decline subsequent to blood sampling (N=237; independent samples t-test, p=0.01).

Table 4.2 - Discovery-phase rapid and non-rapid progressors AD dementia subjects

	Rapid progressors (n=22)	**Non-rapid progressors (n=29)**
Gender (M/F)	9/13	11/18
Age (years)	76 (7.1)	79 (6.8)
Disease duration (years)	4.1 (3.3)	5.0 (4.0)
MMSE	20.7 (4.3)	20.9 (5.2)
Rate of decline in ADAS-Cog score	7.95 (5.2)§	-3.3(4.5)

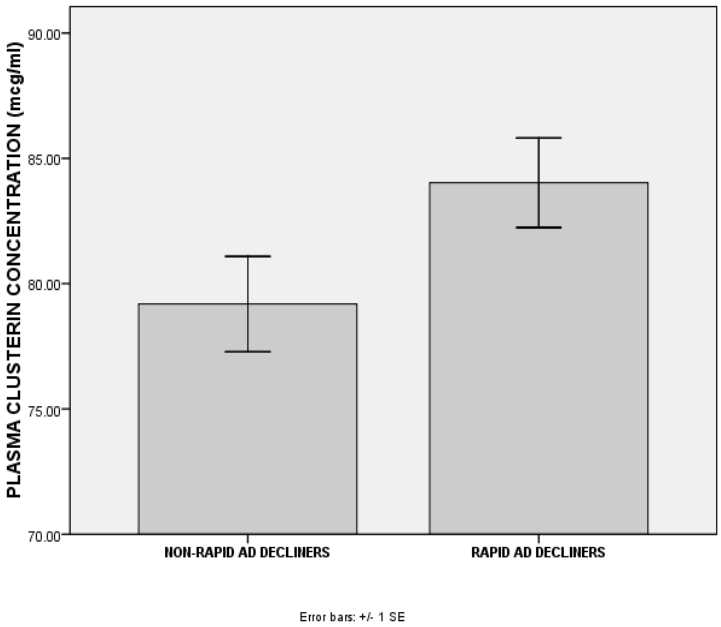
Values are expressed as mean ±(SD)
§ Differs from non-rapid decliners; p<0.001

Table 4.3 - Validation phase: AD dementia fast vs. slow decliners- combined ART and AddNeuromed Cohorts

	Retrospective decline		**Prospective decline**	
	Fast decliners (n=219)	Slow decliners (n=125)	Fast decliners (n=115)	Slow decliners (n=122)
Sex (M/F)	74/145	54/71	43/72	47/75
Age (years)	78.0 (6.2)	77.7 (6.4)	77.7 (6.3)	77.5 (6.4)
Rate of decline in MMSE per year	4.5 (2.7)	1.1 (1.0) *	5.0 (3.2)	-0.9 (2.0) *
Disease duration (years)	3.9 (2.4)	6.4 (3.8) *	4.7 (3.3)	4.0 (3.3)

Values are expressed as mean ± (SD); * p<0.001

Figure 4.2 - AD dementia patients with a rapid retrospective progression rate have significantly increased clusterin concentration relative to slow progressors



4.1.4 Discussion

Increased levels of plasma clusterin were found in rapid decliners compared to non-rapid decliners.

In 1990, mRNA for clusterin was initially found to be significantly elevated in AD affected brain regions when compared to control brains (May et al., 1990). In situ analysis revealed that clusterin was expressed in pyramidal neurons as well as in nonpyramidal cells of hippocampus and entorhinal cortex (May et al., 1990). Clusterin has been found in the frontal cortex and hippocampus of post-mortem AD brains (Lidstrom et al., 1998). The analysis of the clusterin level in CSF has proved difficult since its glycosylation level can change (Nilselid et al., 2006) and clusterin can also form complexes with Aβ peptides and fibrils (Ghiso et al., 1993). Initial studies did not find any difference between control and AD patients (Suzuki et al., 2002), but more recent studies using modern techniques have revealed that the level of clusterin protein in CSF is significantly increased in AD dementia patients (Sihlbom et al., 2008, Ghiso et al., 1993).

This study which used a combined proteomic and neuroimaging approach, showed plasma clusterin was positively associated with brain atrophy in the hippocampus and entorhinal cortex, baseline disease severity, and rapid clinical progression in dementia due to AD, suggesting it as a possible plasma biomarker of AD (Thambisetty et al., 2010). This result was confirmed in transgenic mice that had marked cerebral Aβ deposition and cognitive defects (Thambisetty et al., 2010).

Clusterin and its role as an amyloid chaperone is further discussed in chapter 6, section 6.4.1.

Association of Plasma Clusterin Concentration With Severity, Pathology, and Progression in Alzheimer Disease

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Context: Blood-based analytes may be indicators of pathological processes in Alzheimer disease (AD).

Objective: To identify plasma proteins associated with AD pathology using a combined proteomic and neuroimaging approach.

Design: Discovery-phase proteomics to identify plasma proteins associated with correlates of AD pathology. Confirmation and validation using immunodetection in a replication set and an animal model.

Setting: A multicenter European study (AddNeuroMed) and the Baltimore Longitudinal Study of Aging.

Participants: Patients with AD, subjects with mild cognitive impairment, and healthy controls with standardized clinical assessments and structural neuroimaging.

Main Outcome Measures: Association of plasma proteins with brain atrophy, disease severity, and rate of clinical progression. Extension studies in humans and transgenic mice tested the association between plasma proteins and brain amyloid.

Results: Clusterin/apolipoprotein J was associated with atrophy of the entorhinal cortex, baseline disease severity, and rapid clinical progression in AD. Increased plasma concentration of clusterin was predictive of greater fibrillar amyloid-β burden in the medial temporal lobe. Subjects with AD had increased clusterin messenger RNA in blood, but there was no effect of single-nucleotide polymorphisms in the gene encoding clusterin with gene or protein expression. *APP/PS1* transgenic mice showed increased plasma clusterin, age-dependent increase in brain clusterin, as well as amyloid and clusterin colocalization in plaques.

Conclusions: These results demonstrate an important role of clusterin in the pathogenesis of AD and suggest that alterations in amyloid chaperone proteins may be a biologically relevant peripheral signature of AD.

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Author Affiliations are listed at the end of this article.

Peripheral compartments including blood and cerebrospinal fluid exhibit signals reflecting neuropathological changes in Alzheimer disease (AD).[1,2] In cerebrospinal fluid, these include a decrease in amyloid-β peptide (Aβ) and an increase in total and phosphorylated tau concentrations,[3] reflecting amyloid sequestration as plaques and neurofibrillary degeneration, respectively.[4,5] Similarly, while numerous articles suggest that plasma concentrations of several metabolites and proteins might represent responses to neuropathological changes in AD,[6-11] these findings have not been conclusively replicated.[12] A limitation of such studies may be their reliance upon demonstrating changes between affected and unaffected people, a design of study that might identify secondary changes lacking relevance to core disease biology.

Advances in methods such as proteomics present a further challenge in case-control studies, often generating data showing numerous analytes differentially expressed in AD patients. However, validating these results with alternative methods in independent patient

populations has been difficult.[13,14] These studies also ignore the clinical heterogeneity in disease progression in AD, wherein some patients show rapid cognitive decline, while others remain relatively stable and/or progress slowly.[15,16]

We applied mass spectrometry–based proteomics to discover plasma proteins associated with disease, using brain atrophy in AD as well as rapid clinical progression, rather than binary distinction between case and control. As a proxy measure of in vivo pathology, we used structural neuroimaging of atrophy in the hippocampus and entorhinal cortex (ERC), 2 components of the medial temporal lobe (MTL) that show early pathological changes in AD.[17] For rate of clinical progression, we used both retrospective and prospective measures of cognitive decline. We initially performed 2 independent discovery-phase studies using proteomic analysis of plasma in separate groups of subjects. In the first, we sought proteins that reflect hippocampal atrophy in mild cognitive impairment (MCI) and established AD. In the second, we identified proteins differentially expressed in rapidly progressing AD patients relative to those with a less aggressive disease course. Our aim was to identify plasma proteins common to both paradigms, followed by replication using quantitative immunoassays such as enzyme-linked immunosorbent assay (ELISA) in a large independent cohort of AD, MCI, and control subjects. **Figure 1** illustrates the design of this study.

METHODS

SUBJECTS AND SAMPLES

We used samples from 2 studies: the Alzheimer Research Trust–funded cohort at King's College London (KCL-ART)[7] and the AddNeuroMed study.[18] The KCL-ART study, which began in 2001, includes a cohort of people with AD and MCI[19] and healthy elderly individuals. All subjects are white UK citizens with grandparents born in the United Kingdom and are assessed annually. AddNeuroMed is a cross-European cohort; AD cases are assessed at 3-month intervals in the first year and annually thereafter; MCI and control groups are assessed annually. All subjects are white Europeans recruited from 6 centers in the United Kingdom, France, Italy, Finland, Poland, and Greece. Standardized assessments include demographic and medical information; cognitive assessment, including the Mini-Mental State Examination (MMSE) (both studies; all subjects), Alzheimer Disease Assessment Scale–cognitive subscale (ADAS-cog) (AddNeuroMed only), and Consortium to Establish a Registry for Alzheimer's Disease battery; and scales to assess function, behavior, and global levels of severity, including the Clinical Dementia Rating. Cases with probable AD (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association [NINCDS-ADRDA] criteria) and amnestic MCI were identified as previously described[7] and evaluated with a standardized assessment shown to have high diagnostic validity.[20] Cases with amnestic MCI were defined as having subjective memory complaints, Clinical Dementia Rating scores of less than 1, and evidence of objective memory impairment using the Consortium to Establish a Registry for Alzheimer's Disease delayed word list recall (–1.5-SD cutoff). Normal elderly controls, defined as having no evidence of cognitive impairment (MMSE score >28), were recruited systematically from primary care patient lists in the KCL-ART study and from both primary care services and elsewhere in the AddNeuroMed study. Blood samples were collected and stored as previously described.[7,18] In total, we studied 95 and 689 subjects in discovery and validation studies, respectively, with an additional 60 subjects from the Baltimore Longitudinal Study of Aging (eTables 1-4, available at http://www.archgenpsychiatry.com).[21] Ethical approval was obtained in each of the participating countries.

NEUROIMAGING

Magnetic Resonance Imaging Data Acquisition

In the KCL-ART study, whole-brain coronal 3-dimensional spoiled-gradient recalled images (repetition time=14 milliseconds, echo time=3 milliseconds, $256\times192\times124$ acquisition matrix, 1.5-mm slices) were obtained on a GE Signa 1.5-T neuro-optimized magnetic resonance system. In the AddNeuroMed study, whole-brain sagittal 3-dimensional magnetization-prepared rapid acquisition gradient echo images (repetition time=8.6 milliseconds, echo time=3.8 milliseconds, 256×192 acquisition matrix, 180×1.2–mm slices) were obtained on a 1.5-T magnetic resonance system at each of the 6 centers. Quality control was undertaken using the ADNI Magphan phantom and 2 volunteers who visited each of the centers, ensuring compatibility across the study. Thickness of the ERC was calculated with Freesurfer using a cortical reconstruction technique.[22,23]

^{11}C–Pittsburgh Compound B Positron Emission Tomographic Studies

Dynamic ^{11}C–Pittsburgh Compound B (^{11}C-PiB) positron emission tomographic (PET) studies (37 time frames across 90 minutes) were acquired in 3-dimensional mode on a GE Advance scanner immediately after intravenous bolus injection of approximately 5.55×10^8 Bq (15 mCi) of ^{11}C-PiB. Dynamic images were reconstructed using filtered back projection with a ramp filter (image size, 128×128; pixel size, 2×2 mm; slice thickness, 4.25 mm), yielding a spatial resolution of about 4.5 mm full width at half maximum at the center of the field of view. Parametric images of distribution volume ratios were calculated by simultaneously fitting a reference tissue model using linear regression and spatial constraint with the cerebellum as a reference region.[24,25] The SPM5 program (Statistical Parametric Mapping 5; Wellcome Department of Imaging Neuroscience, London, England) was used to investigate the association between clusterin and medial temporal ^{11}C-PiB retention (significance threshold of $P\leq.05$, with a spatial extent of 25 voxels). Based on a priori hypotheses in light of our results on the association between ERC atrophy and clusterin concentration in AD, a restricted search of the MTL was performed using the regional definition from the WFU PickAtlas.[26]

PROTEOMICS

Two-dimensional gel electrophoresis and liquid chromatography coupled to tandem mass spectrometry (LC-MS-MS) were performed as previously described.[7] Gels were analyzed using image analysis software (either Melanie 2-D or Progenesis SameSpots version, 3.0, Nonlinear Dynamics). Protein spots of interest were excised, washed, digested in gel with trypsin, and analyzed by LC-MS-MS.[7] Mass spectral data were processed into peptide peak lists

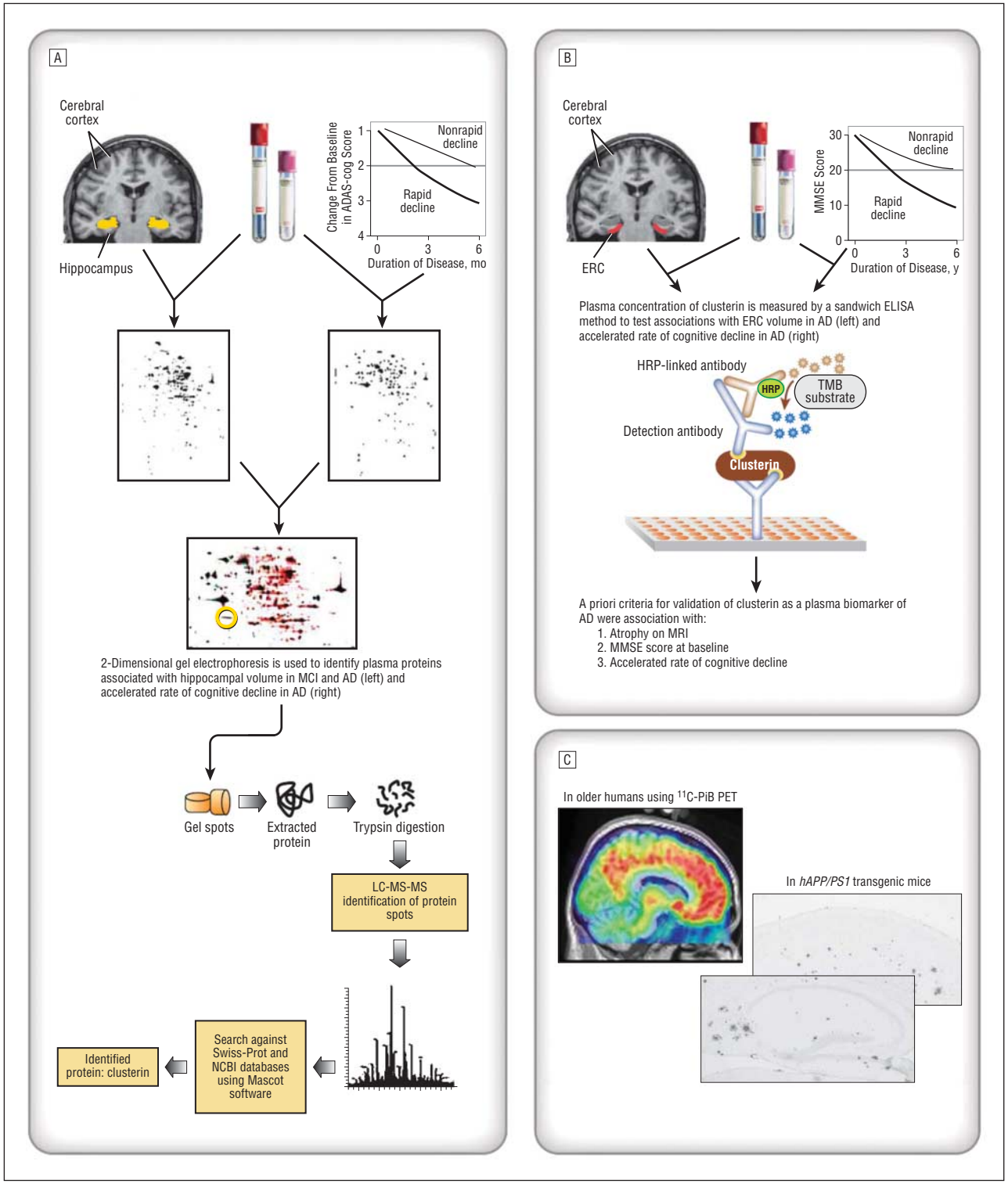


Figure 1. Study design. Schematic diagram of the design of discovery- (A) and validation- (B) phase studies for the identification of blood-based Alzheimer disease (AD) biomarkers associated with both in vivo disease pathology as well as rate of disease progression. C, Association of plasma clusterin concentration with brain amyloid burden was tested in both nondemented older humans and a transgenic mouse model of AD. ADAS-cog indicates Alzheimer Disease Assessment Scale–cognitive subscale; ELISA, enzyme-linked immunosorbent assay; ERC, entorhinal cortex; HRP, horseradish peroxidase; LC-MS-MS, liquid chromatography coupled to tandem mass spectrometry; MCI, mild cognitive impairment; MMSE, Mini-Mental State Examination; MRI, magnetic resonance imaging; NCBI, National Center for Biotechnology Information; TMB, tetramethylbenzidine; and ^{11}C-PiB PET, ^{11}C–Pittsburgh Compound B positron emission tomography.

and searched against the Swiss-Prot Database using Mascot software (Matrix Science, London, England).

For validation experiments, plasma clusterin concentration was assayed by a commercially available ELISA kit (Human Clusterin ELISA, RD194034200R; Biovendor Laboratory Medicine Inc, Modric, Czech Republic). Samples were run in duplicate. Coefficient of variation of the ELISA for all studies overall was 3.5% (baseline data, 3.7%; follow-up data, 3.5%; and BLSA substudy, 3.1%).

GENOMICS

Gene Expression of Clusterin

Approximately 2.5 mL of venous blood was collected into a PAXgene tube for each subject at the baseline visit, processed according to the manufacturer's instructions, and stored at –20°C overnight prior to –80°C storage. RNA was extracted using the PAXgene Blood RNA kit according to the manufacturer's instructions. Samples were assessed for yield using a spectrophotometer and quality using the RNA 6000 Pico Chip on the Agilent Bioanalyser. Samples with an RNA integrity number greater than 7.0 were used for polymerase chain reaction (PCR) assays.

Using the Quantitect Reverse Transcription kit (Qiagen), 500 ng of RNA was reverse transcribed to complementary DNA in a 40-µL reaction and subsequently diluted to 200 µL. Reverse transcriptase–PCR reactions were performed in 384-well plates in the 7900HT Fast Real-Time PCR machine (Applied Biosystems, Foster City, California). The geNORM housekeeping selection kit (Primer Design Ltd, Southampton, England) was used to assay 12 housekeeping genes in a subset of the samples. Using NormFinder software, the 2 most stable genes for normalization were determined to be *SF3A1* and *ATP5B*. Samples were assayed in duplicate, and a standard curve of known copy number was run on each plate for clusterin, *SF3A1*, and *ATP5B*. Data were nonparametric and were therefore log transformed.

Clusterin Genotyping

Tagger software (http://www.broad.mit.edu/mpg/tagger/) identified 7 single-nucleotide polymorphisms (SNPs) (rs9331908, rs11136000, rs867231, rs867230, rs9331888, rs9314349, and rs484377) that captured more than 90% of variation in the clusterin gene. Genotypes were determined using a TaqMan allele-specific assay (Applied Biosystems). The PCR amplifications were performed on an ABI PRISMR 7000 Sequence Detection System (Applied Biosystems). A total of 946 individuals (358 AD subjects, 373 controls, and 215 MCI subjects) were genotyped for the 7 SNPs.

TASTPM Transgenic Mouse Model Experiments

Heterozygote transgenic mice overexpressing hAPP695swe (*Tas10*) and presenilin 1 M146V mutations (*Tpm*) were generated as previously described.[27] Western blot analysis of clusterin was performed in plasma samples at 6 months using an anti–apolipoprotein J mouse polyclonal antibody (Abcam AB349-50; 1:5000). For immunohistochemistry, antigen retrieval was undertaken as described previously.[27,28] Primary antibodies were 1E8 (pan-Aβ), 20G10 (Aβ42; GlaxoSmithKline; 1:1000), and anticlusterin (R&D Systems goat polyclonal AF2747; 1:20 000). Images were captured at ×4 magnification on an Axioscope microscope and analyzed by Image J software to generate percentage Aβ or clusterin load. Animal experiments were conducted according to the Council of Europe guidelines.

STATISTICAL ANALYSIS

Discovery-phase proteomic data were analyzed by partial least-squares regression using SIMCA-P, version 8.0. Spot data were scaled to unit variance and $\log_{10}$ transformed where appropriate. Observations with greater than 50% missing values were excluded. Partial least-squares discriminant analysis was used to derive a panel of protein spots that discriminated between rapidly and slowly declining AD groups.

Validation-phase protein data were examined using SPSS, version 17. Covariates were chosen in cases in which such variables were significantly different between the groups of interest or in which they were likely to influence the dependent variable. To test associations between plasma clusterin concentration and ERC thickness, partial correlation analysis was performed with age and sex as covariates. In analyzing associations between MMSE score and plasma clusterin concentration, partial correlation was performed with age as a covariate. While testing differences in clusterin concentration between rapidly and nonrapidly declining AD patients, age and sex were not included as covariates because they were not significantly different between the 2 groups. However, duration of disease was significantly different between these groups (retrospective analysis) and was therefore included as a covariate in an analysis of covariance (ANCOVA) model. In the prospective analysis, there was no significant difference in disease duration between rapid and nonrapid decliners, and clusterin concentration between these groups was therefore compared using an independent samples *t* test. Linear regression adjusting for disease status, age, sex, and *APOE* ε4 status was performed to investigate the association between *CLU* SNPs and clusterin plasma levels and to examine the relationship between *CLU* messenger RNA (mRNA) and disease. Image analysis is described in the relevant sections. All other statistical analyses were performed using SPSS, version 17, and are described in the text.

RESULTS

PROTEOMIC IDENTIFICATION OF PLASMA PROTEINS ASSOCIATED WITH HIPPOCAMPAL ATROPHY AND RAPID CLINICAL PROGRESSION IN AD

To identify plasma proteins associated with disease as reflected by cerebral atrophy, we first performed a discovery-phase proteomics experiment using 2-dimensional gel electrophoresis and LC-MS-MS, with hippocampal atrophy as the independent variable. We analyzed samples from 44 subjects from the KCL-ART cohort, representing a continuum of disease (27 individuals with mild to moderate AD and 17 with MCI; eTable 1). Bivariate correlation of integrated optical densities of spots detected by 2-dimensional gel electrophoresis revealed 13 spots that were significantly associated with hippocampal volume ($r \geq \pm 0.35$, $P < .05$). Subsequently, using partial least-squares regression,[29] a method suited to analysis of proteomic data in which colinearity among predictor variables is common, a model with 2 components was fitted to the hippocampal volume data. This was constituted by 8 of the 13 spots which, together, explained 34% of the variance (R2Y, ie, explained variance in the outcome variable) in hippocampal volume. Using LC-MS-MS, we identified these 8 spots as complement C3, γ-fibrinogen, serum albumin, complement factor I, clusterin (in 2 spots), α_1-macroglobulin, and serum amyloid P (**Figure 2**). We then performed a second discovery-phase experiment in an independent set of samples in 51 carefully matched (for age, sex, severity at the time of blood sampling, and cholinesterase inhibitor treatment [all were taking the drug]) AD subjects from the AddNeuroMed cohort who we could divide into fast (n = 22) or slow (n = 29) progressors based on their annualized rate of cognitive decline (eTable 1). We defined a priori fast de-

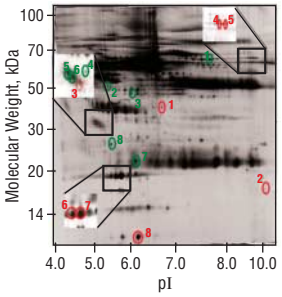
cline as a fall of 2 or more points on the ADAS-cog scale during 6 months. A partial least-squares discriminant analysis model distinguishing the rapidly from the slowly progressing AD groups was constituted by the integrated optical densities of 27 silver-stained 2-dimensional gel electrophoresis spots. Of these, 8 were well defined, discrete, and present in all 51 gels and were identified by LC-MS-MS. These spots contained complement component C4 (in 3 spots), complement C8, clusterin, apolipoprotein A1 (in 2 spots), and transthyretin (Figure 2).

CLUSTERIN AND ATROPHY OF THE ERC, SEVERITY OF COGNITIVE IMPAIRMENT, AND SPEED OF PROGRESSION IN AD

Only 1 protein was common to both discovery-phase studies: clusterin. We therefore sought to confirm this finding in a large cohort of 689 subjects, including 344 from the AddNeuroMed study (119 with AD, 115 with MCI, and 110 controls) and 345 (all with AD) from the KCL-ART cohort (eTable 2). We used atrophy in the ERC as an alternative measure of disease pathology (Figure 1). The 689 validation-phase subjects included the 95 subjects in the discovery phase albeit with entirely different analytical measures in the 2 studies.

Confirming the discovery-phase study, we observed a trend toward association between clusterin concentration and ERC atrophy in the combined AD and MCI cohort (n=219; $R=-0.12$, $P=.06$) after covarying for age and sex. This relationship was driven primarily by a highly significant association between ERC atrophy and clusterin concentration in AD patients (n=113; $R=-0.30$, $P=.001$). We also correlated plasma clusterin concentration with MMSE score—a measure of cognition available in 576 subjects with MCI and AD—and again found a highly significant negative correlation ($r=-0.22$; $P<.001$, age as a covariate).

We then compared clusterin levels in rapidly declining AD patients relative to slow decliners using both retrospective and prospective measures of decline relative to the time of blood sampling (Figure 1 and eTable 2). Retrospective decline was estimated from the duration of disease and the MMSE score at the point of blood sampling, allowing the annualized fall in MMSE score to be calculated. We used MMSE score, as the ADAS-cog score was not available in all subjects, and defined fast decline as a fall of 2 points or more during a 1-year period relative to the time of blood sampling. Prospective decline was directly measured as the fall in MMSE score 1 year after blood sampling. We observed a significant increase in clusterin concentration in AD patients with accelerated cognitive decline prior to blood sampling (n=344; ANCOVA, $t_{341}=3.40$, $P<.001$, duration of disease as covariate) (**Figure 3**A) and an increase in clusterin concentration in AD patients with faster cognitive decline subsequent to blood sampling (n=237; independent samples t test, $P=.01$) (Figure 3B). Cox proportional regression analysis showed that higher plasma clusterin concentration was associated with a greater risk of rapid cognitive decline 1 year after blood sampling (Figure 3C). We then performed an analysis of variance (age and sex as covariates) between AD, MCI, and control groups in the entire sample to test for differences in plasma clusterin concentration. There were no significant differences: AD, 82.4 ng/mL (SD, 25.6 ng/mL; n=336); MCI, 77.6 ng/mL (SD, 22.5 ng/mL; n=222); and control subjects, 82.2 ng/mL (SD, 23.8 ng/mL; n=385). Finally, we compared differences in plasma clusterin concentration between *APOE* ε4 carriers and noncarriers (independent samples t test) in the combined cohort of AD, MCI, and control subjects and did not find any significant difference.



Protein	Accession No.
1, Complement C3	P01024
2, γ-Fibrinogen	P02679
3, Serum albumin	P02768
4, Complement factor I	P05156
5, Clusterin	P10909
6, Clusterin	P10909
7, Serum amyloid P	P02743
8, α_1-Macroglobulin	P02760

Protein	Accession No.
1, Complement C4a	P0C0L4
2, Complement component C8	P07360
3, Clusterin	P10909
4, Complement C4a	P0C0L4
5, Complement C4a	P0C0L4
6, Apolipoprotein A1	P02647
7, Apolipoprotein A1	P02647
8, Transthyretin	P02766

Figure 2. Gel-based proteomic discovery-phase studies. Proteomic identification of plasma proteins associated with hippocampal volume in subjects with Alzheimer disease (AD) and mild cognitive impairment (MCI) and those associated with fast AD progression (bottom panel). A representative 2-dimensional gel electrophoresis gel is shown with spots outlined in green denoting proteins associated with hippocampal volume in AD and MCI and those in red highlighting proteins associated with fast AD progression.

CLUSTERIN AND FIBRILLAR Aβ BURDEN IN THE ERC IN NONDEMENTED OLDER INDIVIDUALS

Because high clusterin levels are associated with brain atrophy and a more rapid rate of cognitive decline in AD patients, we hypothesized that increased clusterin concentration might be an antecedent marker of pathology in otherwise normal older individuals. We tested this hypothesis in participants of the Baltimore Longitudinal Study of Aging who had stored samples of plasma and underwent PET imaging of fibrillar Aβ burden with ^{11}C-PiB (n=60; eTable 3). Although all participants were nondemented at the time of the PiB-PET study, a range of in vivo amyloid burden is observed in cognitively normal individuals[30] and increased amyloid deposition may represent the earliest phase of AD pathology in these subjects. Measuring plasma clusterin concentration from samples collected 10 years before the PiB-PET studies, we investigated associations between clusterin concentration and subsequent development of in vivo fibrillar amyloid burden.

We conducted a directed search of significant associations between clusterin and MTL PiB values using the MTL region defined by the WFU PickAtlas[26] and the SPM5 multiple regression module, adjusting for age and sex. These results indicated that higher antecedent clusterin concentrations were associated with greater PiB retention in bilateral ERC; it was higher on the right (right ERC, $P=.009$; and left ERC, $P=.03$) (**Figure 4**A). This suggests that

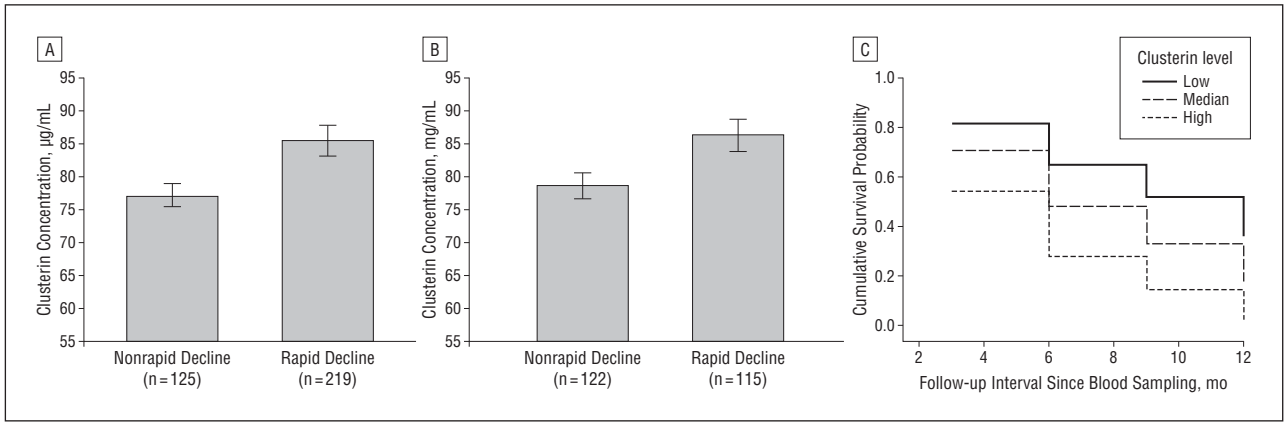


Figure 3. Increased concentration of plasma clusterin and rate of clinical progression in Alzheimer disease (AD). Patients with AD with a rapid progression rate, measured prior to blood sampling (A) and 1 year after blood sampling (B) have significantly increased clusterin concentration relative to slow progressors. C, High levels of clusterin are associated with a significantly greater risk of accelerated cognitive decline subsequent to blood sampling. Patients with AD (n=204) were assigned a prognostic index derived as their plasma clusterin concentration multiplied by its corresponding regression coefficient (β) in a Cox proportional regression analysis. C, Cumulative hazard functions for the effect of the prognostic factor (ie, plasma clusterin concentration) on the survival probability, ie, maintaining a nonaggressive clinical course (decline in Mini-Mental State Examination score ≤2 points per year). The cumulative survival functions represent estimated survival probabilities for 3 representative AD patients with the lowest (5.87 ng/mL), median (76.84 ng/mL), and highest plasma clusterin (159 ng/mL) concentrations showing that an AD patient with the highest clusterin concentration has the lowest probability of maintaining a nonaggressive clinical course 1 year after sampling. The reported hazard ratio for a 10-ng/mL rise in plasma clusterin concentration for risk of becoming a rapid AD decliner was 1.071 (95% confidence interval; 1-1.147; $P=.05$).

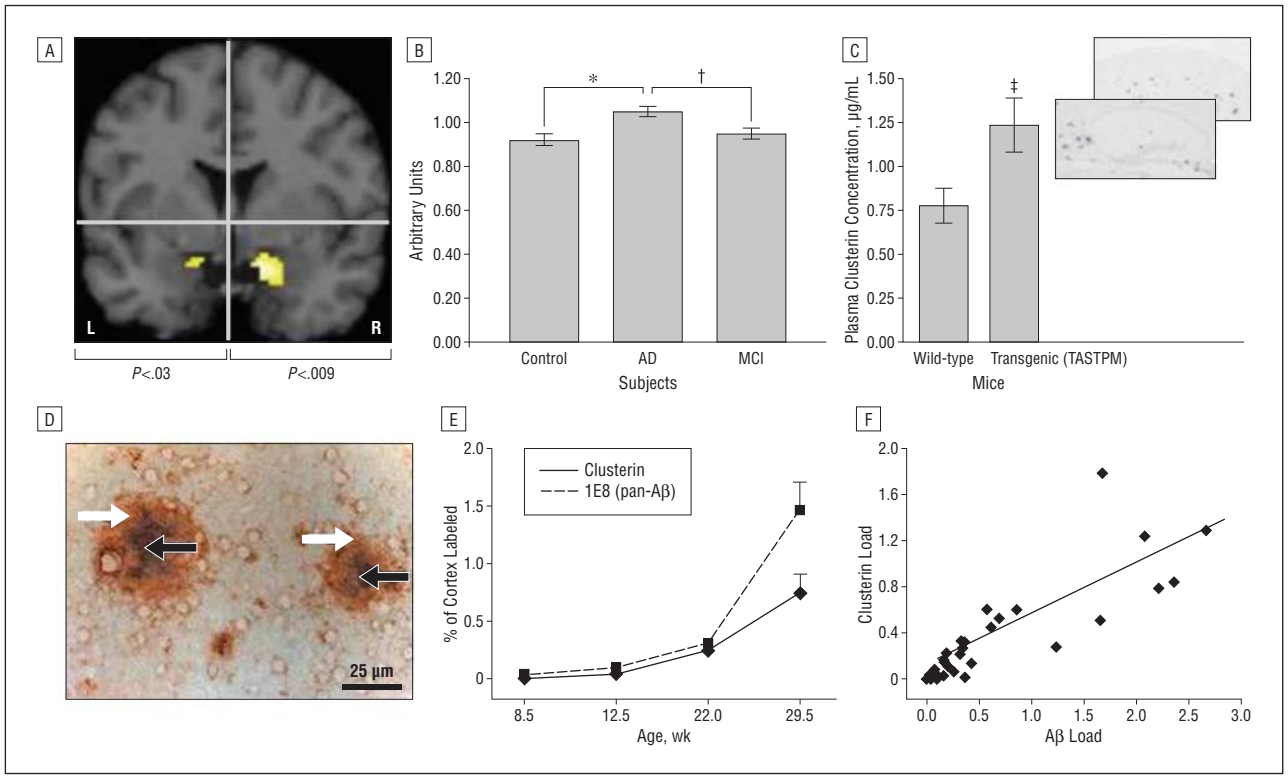


Figure 4. Clusterin expression and amyloid pathology. A, Clusterin is an antecedent biomarker of in vivo fibrillar amyloid-β (Aβ) burden in the entorhinal cortex in nondemented older individuals (n=60). SPM analysis shows correlation between plasma clusterin concentration and ^{11}C–Pittsburgh Compound B (^{11}C-PiB) uptake controlling for age and sex ($P<.05$, uncorrected). Highlighted areas denote regions in the entorhinal cortex of both hemispheres that show significant association with plasma clusterin concentration 10 years prior to the PiB positron emission tomographic scans. B, Gene expression of clusterin is altered in Alzheimer disease (AD). Clusterin messenger RNA levels are significantly elevated in blood cells from AD patients (n=182) relative to healthy controls (n=179, *$P<.001$) and subjects with mild cognitive impairment (MCI) (n=207, †$P=.008$) after correcting for age. C, Transgenic TASTPM mice (n=10) overexpressing both human *APP* and *PS1* genes have significantly higher plasma concentration of clusterin relative to wild-type litter mates (n=10) at 6 months of age ($P=.02$). Inset shows hippocampal and cortical amyloid plaques in a 6-month-old TASTPM mouse stained by a monoclonal antibody against Aβ1-42. Wild-type mice show no amyloid pathology at this age (not shown). ‡Statistically significant. D, Representative photomicrograph of cortical amyloid plaques in a 6-month-old TASTPM mouse. A close association is observed between Aβ within amyloid plaques (black arrows indicate monoclonal antibody to Aβ42; gray-black labeling, diaminobenzidine) and clusterin (white arrows indicate polyclonal antibody; brown-labeled with Novared). Colors have been slightly enhanced digitally for illustrative purposes. E, TASTPM mice show age-dependent increases in cortical Aβ and clusterin load as determined by quantitative image analysis of immunohistochemical labeling. F, TASTPM mice demonstrate a highly significant ($P<.001$) correlation between Aβ and clusterin load (n=39, male and female mice, 8-30 weeks of age). B, C, and E, Error bars indicate standard error.

increased plasma concentration of clusterin, even in nondemented older individuals, predicts a greater extent of fibrillar amyloid burden in the ERC, the same region where we have also demonstrated robust association with atrophy in subjects with MCI and AD.

GENE EXPRESSION OF CLUSTERIN IS ALTERED IN AD

To investigate the mechanisms underlying the associations between plasma concentration of clusterin and both imaging measures of atrophy and accelerated clinical progression, we measured clusterin mRNA levels in blood cells from AD patients (n=182), MCI subjects (n=179), and controls (n=207) (eTable 4). Diagnosis had a significant effect on clusterin gene expression (ANCOVA, $df=2$, $P<.001$, age as a covariate). Pairwise comparisons between the 3 groups showed significantly higher clusterin gene expression in AD patients than in MCI and control subjects ($P=.008$ and $P<.001$, respectively, Bonferroni adjustment for multiple comparisons) (Figure 4B). Sex and presence of the *APOE* ε4 allele did not have a significant effect on clusterin mRNA levels. We did not observe a significant association between clusterin mRNA in blood cells and plasma concentration of clusterin protein nor did we find a correlation between plasma mRNA levels and either MMSE score or rate of decline in MMSE score within groups or with atrophy on neuroimaging.

LACK OF EFFECT OF VARIATION IN THE CLUSTERIN GENE ON PERIPHERAL CLUSTERIN EXPRESSION

We did not observe significant effects of the 7 clusterin gene SNPs on either clusterin mRNA expression in blood cells or plasma concentration of clusterin (eTable 5 and eTable 6). The SNPs analyzed included those reported on in the recent large Genome-Wide Association Studies to be associated with risk of sporadic AD.[31,32]

PLASMA CONCENTRATION OF CLUSTERIN IN TRANSGENIC MICE WITH PLAQUE PATHOLOGY

To extend our findings on the association of clusterin with brain amyloid deposition, we examined its plasma concentration in a transgenic mouse model of AD. TASTPM mice overexpress the hAPP695swe and presenilin 1 M146V mutations, resulting in overproduction of human amyloid precursor protein,[27] and mimic various hallmarks of AD including amyloid plaques as well as cognitive and behavioral deficits.[27,28] In light of our magnetic resonance imaging data in AD patients and PiB-PET results in nondemented older individuals, we hypothesized that plasma clusterin concentration in transgenic TASTPM mice would be higher than wild-type controls. As predicted, we observed a significantly greater plasma concentration of clusterin ($P=.02$, independent samples *t* test) in 6-month-old transgenic TASTPM mice (n=10) relative to wild-type litter mates (n=10) (Figure 4C). Previous studies have established both marked cerebral Aβ deposits as well as cognitive deficits in TASTPM mice at this age relative to wild-type litter mates.[27,28]

BRAIN CLUSTERIN AND AMYLOID IN A TRANSGENIC MOUSE MODEL OF AD

Using double-labeling immunohistochemistry, we demonstrated that cortical plaques in TASTPM mice contained both Aβ and clusterin (Figure 4D). Finally, we established the close association between Aβ and clusterin by showing that both cortical Aβ burden and clusterin deposition increase with age in TASTPM mice (n=9-11) (Figure 4E) and that there is a highly significant correlation ($F_{1,37}=107.57$, $P<.001$, adjusted $R^2=0.737$) between cortical Aβ and clusterin load (Figure 4F).

COMMENT

We have combined a novel proteomic and neuroimaging approach to establish that plasma concentration of clusterin is associated with in vivo pathology, disease severity, and clinical progression in patients with AD. The primary outcomes in our discovery-phase studies were association with both atrophy of the MTL and the rate of progression of cognitive decline. In the discovery phase, we used hippocampal atrophy derived from manual tracing of the hippocampal formation from magnetic resonance imaging, and in the much larger validation phase, from automated regional analysis of the ERC, an adjacent region of the MTL and the site of earliest pathology in AD.

Hippocampal atrophy is an early event in the pathogenesis of AD, is associated with an increased risk of conversion from MCI to AD, and may even precede the development of cognitive decline.[33,34] Cerebrospinal fluid levels of phosphorylated tau correlate with hippocampal volume, indicating that this measure reflects an integral feature of AD pathology.[35] Moreover, decreased hippocampal volume in AD patients is associated with neuronal loss, confirming its validity as a marker of neurodegeneration.[35] A second independent outcome variable in the discovery-phase studies was rate of cognitive decline, derived as a measure of decrease in the ADAS-cog scores during a 6-month interval in AD patients. Using this measure, we dichotomized AD patients as fast and slow decliners, an approach previously shown to predict long-term prognosis in AD.[36]

Only clusterin was associated both with hippocampal atrophy in AD and MCI subjects and with fast progressing, or more aggressive, AD. Evidence from human cerebrospinal fluid, postmortem brain, and transgenic animal models suggests a plausible link between clusterin and AD pathology.[37-40] We therefore sought to confirm the association of clusterin with AD pathology, severity, and progression in a much larger validation-phase study.

We confirmed highly significant associations of plasma clusterin concentration with atrophy of the ERC ($P=.001$), MMSE score ($P<.001$), and rate of progression in AD ($P<.001$). We also demonstrated a significantly greater risk of subsequent accelerated cognitive decline associated with increased concentration of clusterin in patients with AD and, in normal individuals, with subse-

quent deposition of fibrillar Aβ in the ERC. Our finding of raised plasma clusterin concentration 10 years before fibrillar Aβ deposition in the brain in normal elderly individuals suggests that clusterin is raised very early, possibly as an etiopathological event, and is not simply a reaction to other pathology in AD. The observation that clusterin mRNA is significantly increased in blood cells in AD suggests that the observed changes in protein levels reflect changes in expression in disease and not, for example, altered turnover. However, the increase in clusterin mRNA in AD patients does not correlate directly with plasma clusterin concentration, suggesting that the primary sources of plasma clusterin that we find predictive of more aggressive disease are organs other than blood cells such as the liver or possibly even the brain. In the course of this study, 2 groups, including one in which we participated, reported from genome-wide studies that polymorphic variation in *CLU*, which encodes clusterin, was associated with AD.[31,32] One possible mechanism for this association would be for the SNPs associated with disease to be modifiers of gene expression. To investigate this, we determined the effect of variations in the clusterin gene on both peripheral mRNA levels and plasma concentration of clusterin protein, including the principal variant associated with disease and 6 other SNPs determined to cover most of the variation in the gene. We did not find significant effects of these SNPs on either peripheral mRNA levels or plasma clusterin concentration, suggesting that our observed association of clusterin protein and mRNA with AD-related pathological processes is independent of genetic variation in the clusterin gene. Our findings raise the possibility of 2 possibly linked mechanisms whereby both altered expression and some other factor in the gene linked to the disease-associated SNPs are active in moderating disease pathology. However, we cannot exclude an effect of genetic variation not examined in this study on clusterin expression or a small effect of *CLU* variation, below the power of our study to detect, on expression. Nonetheless, the finding of association with both genetic variants and, as we now report, gene and protein expression adds considerable weight to the importance of clusterin to AD pathogenesis. It is interesting that we observe clusterin in 2 closely related but distinct spots in the discovery-phase 2-dimensional gel electrophoresis studies. Proteins are components of multiple spots on 2-dimensional gel electrophoresis because of changes in posttranslational modification, complex formation, and splicing changes resulting in different isoforms. It is possible that some of these variations might be associated with disease processes in addition to the overall amount of protein as measured in the validation-phase study. Finally, we confirmed a previous report of significantly higher plasma concentration of clusterin in TASTPM mice overexpressing *APP/PS1* mutations,[41] and we also show that clusterin is closely associated with cortical amyloid plaques, showing an age-dependent concomitant increase with brain amyloid burden.

Previous studies suggest that clusterin belongs to a family of extracellular chaperones that regulate amyloid formation and clearance.[42] In vitro experiments show that clusterin regulates amyloid formation in a biphasic manner with low clusterin to substrate ratios enhancing and higher ratios inhibiting amyloid formation, respectively.[43] In mice, in vivo binding of Aβ to clusterin enhances its clearance and efflux through the blood-brain barrier.[44] However, previous studies reporting differences in cerebrospinal fluid clusterin concentration between AD patients and controls have been inconclusive.[39,40] Our findings may have implications for the discovery and characterization of other amyloid chaperone proteins in blood linked to AD pathogenesis. In this context, α_2-macroglobulin has recently been characterized as an amyloid chaperone that inhibits fibril formation.[45,46] In a previous proteomic analysis of plasma, we reported the differential expression of α_2-macroglobulin in AD patients and have also found associations between the plasma concentration of α_2-macroglobulin and hippocampal metabolite abnormalities in AD.[7,47] In this previous study,[7] in addition to α_2-macroglobulin, we also identified components of the complement pathway associated with AD. In the discovery phase of the current study, we note many of the same proteins and also that clusterin may itself play a role in complement activation, suggesting that further examination of this pathway may be useful to identify markers associated with AD.[7]

In summary, we have used a novel proteomic-neuroimaging discovery paradigm in which the primary end points were well-established measures of pathology in the MTL and rate of disease progression. We identified clusterin as a plasma protein associated with disease pathology, severity, and progression in AD. Although these findings do not support the clinical utility of plasma clusterin concentration as a stand-alone biomarker for AD, they reveal a robust peripheral signature of this amyloid chaperone protein that is responsive to key features of disease pathology. Our findings clearly implicate clusterin, but there may well be other proteins in plasma related to the disease process, and indeed our previous studies and those of others suggest this is the case. These results may have wider implications for the identification of other amyloid chaperone proteins in plasma, both as putative AD biomarkers as well as drug targets of disease-modifying treatments.

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4.2 Paper 2

PLASMA TRANSTHYRETIN AS A CANDIDATE MARKER FOR ALZHEIMER'S DISEASE

Velayudhan L et al. Journal of Alzheimer's disease, 2012; 28(2):369-75

4.2.1 My contribution:

Transthyretin (TTR) was another key protein that I had identified in the mass spectrometry-gel based proteomics study in plasma differentiating fast and slow progressors in Alzheimer disease (AD) dementia patients (paper 1, table 1). I carried out a further validation study evaluating this protein in an independent larger cohort of subjects with AD dementia, using quantitative enzyme-linked immunosorbent assays (ELISA), to examine it as a marker of AD dementia progression. Following sample selection, I carried out the ELISA experiments, did detailed analysis and interpretation, and wrote the manuscript for publication. I further carried out the necessary corrections following detailed and anonymous peer review process from the submissions under the guidance of my Supervisors, Dr John Powell and Prof Simon Lovestone. I am the corresponding author for this paper.

4.2.2 Introduction

In the discovery phase experiment, proteins differing in plasma between fast and slow progressors included those previously identified in biomarkers studies – including clusterin, complement proteins and Apolipoprotein A1. Validation for clusterin protein was described in the previous chapter. One novel plasma protein was identified in my discovery experiment – transthyretin.

TTR is a 55 kDa homotetrameric transport protein that is synthesized in the liver and choroid plexus and is present in both blood (3–7 μM) and cerebrospinal fluid (CSF, 0.1–0.4 μM) (Richardson, 2007, Du et al., 2012). An increased level of TTR expression in mouse models of AD has been confirmed by various groups (Tsai et al., 2009, Li et al., 2011). Furthermore, neurons from human AD patients, but not age-matched controls, secrete TTR (Li et al., 2011). The protective effect of TTR against Aβ toxicity has been observed in vitro (Giunta et al., 2005, Costa et al., 2008) and supported by other animal studies. For example, progeny from Swedish mutation of APP (APPSw) mice crossed

with mice engineered to express human TTR performed as well as wild-type and better than APPSw mice in cognitive tests (Buxbaum et al., 2008) and AD mice raised in an enriched environment expressed more TTR and performed better on cognitive tests than those raised in a control environment (Costa et al., 2007). TTR plays an important role in keeping intracerebral proteins such as amyloid fibrils in a soluble form and it might inhibit Aβ aggregation and the formation of senile plaques (Stein and Johnson, 2002b, Choi et al., 2007).

Previous studies have reported decreased TTR levels in CSF of AD patients compared to non-demented controls and in those with severe illness (Merched et al., 1998, Riisoen, 1988, Puchades et al., 2003, Castano et al., 2006, Gloeckner et al., 2008a, Hansson et al., 2009a). Low levels of TTR in CSF have been reported to be AD-specific compared when analysed in samples from 35 subjects with AD, 18 subjects with fronto-temporal dementia (FTD) and 29 non-demented (Hansson et al., 2009a). In a recent report TTR is one of the 6 CSF biomarkers in AD describing six clinic-pathological stages from cognitive normalcy to mild dementia, including stages defined by increased risk of cognitive decline (Perrin et al., 2011).

To confirm the findings from the discovery phase that the plasma transthyretin levels was altered between fast and slow progressors, plasma TTR levels were further tested in a larger, independent AD dementia cohort and correlated with the rate of cognitive decline and severity.

4.2.3 METHODS:

4.2.3.1 Subjects, experiment and classification of AD dementia group for cognitive decline and severity

Subjects were recruited, sampled and assessed as previously described in chapter 4-paper 1 (subjects and samples, 4.1.2.1).

TTR protein was assayed by a commercial ELISA kit (Assaypro- AssayMax Human prealbumin ELISA Kit). The experiment was carried out as per the manufacturer's instruction. Baseline plasma samples of AD subjects in an independent sample set (n=270) from both AddNeuroMed (n=177) and KCL-ART (n=93) collections were run in duplicate. Coefficient of variance was less than 10%.

Cognitive decline was defined using MMSE scores, as this was available for all the subjects and as previously described in chapter 4 (4.1.2.3.1). Briefly, annualized fall in MMSE was calculated from the duration of disease and MMSE at the point of blood sampling and rapid cognitive decliners were defined as subjects with fall of 2 or more points over a period of one year (chapter 4, 4.1.2.3.1). I further defined mild AD as those subjects with probable AD dementia with MMSE scores 20 and above points. Moderate to severe AD dementia (Mod-severe AD) was defined as AD dementia in those subjects with MMSE scores between 0-19. MMSE score change over a period of 6 months post-venepuncture was calculated for prospective cognitive decline.

Western blot analysis was carried out to measure and compare TTR levels between AD subjects and age matched non-demented controls. Equal volumes of plasma from AD (n=90) and controls (n=50) (ART-KCL) were immunoblotted for TTR, in duplicate. A standard pooled sample was loaded in duplicate on each gel, to which each test sample was normalised, and which allowed inter-gel comparisons to be made. When assessing the reproducibility of the duplicate gels, a large positive correlation of 0.84 was obtained (Pearson correlation test).

4.2.3.2 Statistical Analysis

Protein data was analysed using SPSS version 17 (for Windows). Chi-square, student t-test, correlation analysis (Spearman non-parametric test) and non-parametric Mann-Whitney-Wilcoxon test were used to compare the socio-demographics, MMSE test scores and TTR protein between groups: rapid and non-rapid cognitive decliners; and mild and mod-severe AD dementia subjects. Linear regression was performed to determine association between TTR and change in the MMSE scores in 6 months from the baseline.

Characterisation of the ELISA analytical performance is important to establish what type of difference can be detected with confidence in future studies of this biomarker. For this, the within-sample variance was determined. For 320 AD dementia plasma samples run in duplicates for TTR concentration with the commercially available assay (Assaypro), the mean within sample variance between replica samples was 3.1%. So,

the assay was able to detect differences as low as 12% difference in the mean TTR levels between the rapid decliners and non-rapid decliners (lower in rapid decliners).

4.2.4 MAIN RESULTS The main results were as below:

1. TTR levels were significantly ($p=0.004$) reduced in AD compared to NDC, when compared between AD subjects and age matched non-demented controls.
2. When comparing AD subjects by speed of decline, TTR levels were significantly lower in subjects with more rapid cognitive decline ($p= 0.036$) and also in subjects with moderate-severe AD ($p<0.01$) (Mann-Whitney U test).
3. Linear regression analysis showed TTR levels as a better predictor factor for MMSE score change in the six months following venepuncture($p=0.029$), in both adjusted and unadjusted models with variables such as age, gender, duration of illness, baseline MMSE and APOE4 carrier status.

Discussion as in the published paper and also further elaborated in chapter 6, section 6.4.2.

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Plasma Transthyretin as a Candidate Marker for Alzheimer's Disease

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Abstract. Diagnosis of the progressive neurodegenerative disorder Alzheimer's disease (AD) can only definitively be made postmortem. The most promising AD biomarkers identified to date are found in cerebrospinal fluid (CSF). Among these, one of the most interesting candidates is transthyretin (TTR), the carrier of thyroxine and retinol, which also binds with amyloid-β (Aβ), and it has been suggested that it protects against Aβ deposition. A biomarker detectable in plasma would have great diagnostic value and could be of use for determining disease progression and the monitoring of therapeutic efficacy due to its greater accessibility over CSF-based markers. We aimed to validate TTR as a prognostic marker in AD and to determine its relation with cognitive measures. We examined the plasma protein levels of TTR in 90 people with late-onset AD and 50 age-matched non-demented controls (NDC) by immunoblotting and found lower plasma TTR levels in AD compared to NDC ($p = 0.004$). We then quantified plasma TTR by enzyme-linked immunosorbent assays in a larger independent cohort ($n = 270$) including subjects with mild to severe AD. Plasma TTR levels were significantly lower in AD cases with rapid cognitive decline and with severe cognitive impairment. Regression analyses showed plasma TTR levels also predicted cognitive decline over the ensuing 6 months. These data indicate that plasma TTR is a strong candidate AD biomarker that should be included in the development of blood based biomarker panels for disease diagnosis and also suggests that plasma TTR is a marker of disease severity and progression.

Keywords: Alzheimer's disease, cognitive impairment, plasma proteins, transthyretin

Supplementary data available online: http://www.j-alz.com/issues/28/vol28-2.html#supplementarydata06

INTRODUCTION

With the rapidly aging global population, the number of people with dementia is estimated to quadruple worldwide in the next 20 years [1]. Alzheimer's disease (AD) is by far the most common dementia and is progressive in nature. A biomarker to aid the early diagnosis of AD, allowing the use of disease modifying therapies before overt dementia manifests or in the monitoring of disease progression would therefore be of great clinical value.

Considerable progress in the search for biomarkers has been made with markers derived from amyloid

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plaques (amyloid-β (Aβ)) [2] and neurofibrillary tangles (tau and phospho-tau) [3]. The most promising sources for biomarkers in AD are cerebrospinal fluid (CSF) and blood plasma, because compared to brain tissue, these fluids are more easily accessible and, in the CSF, which is in close contact with the central nervous system, where key biochemical changes take place. However, while CSF is a good resource for the study of biomarkers in AD, its clinical application is limited by the relatively invasive nature of the procedure. Blood-based biomarkers have an advantage in that they are suitable for large scale studies, in community settings, with the ease of venepuncture allowing for repeatability in old and frail people and applicable to clinical settings.

Many approaches to identifying factors associated with disease characteristics such as speed of progression, have been employed. These include clinical factors, neuroimaging, genetics and various approaches to discover biomarkers in body fluids. Proteomic studies using CSF and blood have identified potential AD diagnostic markers, distinguishing AD patients from healthy elderly controls and other neurodegenerative disorders [4–6], and other studies have used protein-based studies to identify potential predictive markers in mild cognitive impairment (MCI) [7]. However, few studies have yet sought to go beyond diagnostic markers to identify potential prognostic markers in AD. Here we report the validation of one of the key proteins, transthyretin (TTR), identified from a mass spectrometry-gel based proteomics study in plasma, evaluating them further in larger independent cohorts using immunoblotting and quantitative enzyme-linked immunosorbent assays (ELISA). We investigated whether TTR distinguished AD from healthy controls and also its correlations with the rate of cognitive decline and severity in AD.

MATERIAL AND METHODS

Subjects and samples

The samples used in these analyses came from two studies: AddNeuroMed studies and the Alzheimer's Research Trust cohort, Kings College London (KCL-ART). As a part of the KCL-ART study, people with AD, MCI, and non-demented controls (NDC) have been recruited and sampled from 2001 onwards [8]. All subjects were white Europeans with grandparents born in the UK and underwent assessments annually. The AddNeuroMed project, a European Union study, recruited subjects with AD, MCI, and NDC from 6 centers in the UK, France, Italy, Finland, Poland, and Greece [9]. All subjects were assessed at 3-monthly intervals over a year. Assessments in both the studies included a semi-structured interview for demographics, case history, family history, medical history, and standardized tools used to assess cognition, function, behavior, global severity [8]. Patients with probable AD (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association [NINCDS-ADRDA] criteria) in both the studies were identified as previously described [8] and evaluated with a standardized assessment shown to have high diagnostic validity [11]. Age-matched NDC, defined as having no evidence of cognitive impairment (with a MMSE greater than 28), were recruited systematically from primary care patient lists (KCL-ART study) [8]. The full standardized assessments in both of the studies are similar and included demographic and medical information, scales to assess function, behavior, and global levels of severity including the Cambridge Examination for Mental Disorders of Older People (CAMDEX) [12]; and cognitive assessment including Mini Mental State Examination (MMSE) [13] (both studies; all subjects) and Alzheimer disease assessment scale-Cognitive (ADAS-cog) [14] (AddNeuroMed only) [15, 16]. Peripheral venous blood was collected at baseline (initial assessment) and at subsequent time points, including plasma samples collected in 9 ml EDTA tubes and stored at -80°C according to rigorous standard operating procedures. In total, we studied 50 NDC and 90 AD subjects for immunoblotting (KCL-ART cohort) and 270 AD subjects for ELISA (AddNeuroMed cohort), with an additional 40 subjects (AddNeuroMed cohort) for determining correlation between the two techniques. Ethical approval was obtained from local ethic committees.

Criteria for cognitive decline and severity in AD patients

Cognitive decline was defined using MMSE scores, as this was available for all the subjects and previously described [15, 16]. Briefly, annualized fall in MMSE was calculated from the duration of disease and MMSE at the point of blood sampling and rapid cognitive decliners were defined as subjects with a drop of 2 or more points over a period of one year [15]. We further defined mild AD as those subjects with probable AD with MMSE scores of 20 points and above. Moderate to severe AD (Mod-severe AD) was defined as AD in those subjects with MMSE scores between 0–19.

MMSE score change over a period of 6 months post-venepuncture was calculated for prospective cognitive decline.

Validation of TTR using western blotting and enzyme-linked immunoassay

The discovery phase (mass spectrometry-gel based proteomics) for this study has been previously reported [15]. Briefly, plasma samples from AD subjects (AddNeuroMed cohort) characterized as rapid ($n = 22$) and non-rapid progressors ($n = 29$) were subjected to two-dimensional difference-in-gel electrophoresis (2DGE). PLS-DA model discriminating the fast from slow progressing AD groups was constituted by the integrated optical densities of silver-stained 2DGE spots. Transthyretin was identified as one of the proteins from these well-defined, discrete spots, present in all 51 gels by mass spectrometry LC-MS/MS [15].

Western blot analysis was carried out to measure TTR levels in a sample set of 90 AD subjects and 50 healthy controls (KCL-ART cohort). Plasma samples were diluted (4 μl raw plasma plus 96 μl of PBS containing protease inhibitor cocktail (Complete®, 1836145, Roche Applied Science, Penzberg, Germany) and mixed with 100 μl of 2 × reducing Laemmli sample buffer (S3401, Sigma). Samples were then boiled at 100°C for 5 min, centrifuged at 15,500 g and separated on NuPAGE® (24 well), 4–12% Bis-Tris SDS-polyacrylamide gels (Invitrogen, Paisley, UK). Proteins were electroblotted onto 0.2 μm nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany), blocked in 5% dried skimmed milk in PBS + 0.1% Tween (PBST) and probed with a rabbit anti-human TTR antibody (Dako, Ely, UK) for 2 h at room temperature. Primary antibody immunoreactivity was detected with an anti-rabbit antibody conjugated to a 680 nm fluorophor (Alexis, Invitrogen, Paisley) and visualized on an Odyssey near infrared scanner (LI-COR Biosystems, Nebraska, USA). Densitometric analysis was performed using the Odyssey software v 2.1. All samples were run in duplicate and intensities were normalized to a reference plasma sample run on each gel (also loaded in duplicate) to allow inter-gel comparisons. The densitometric values obtained for each duplicate run were averaged post normalization to the in gel control sample.

To validate the novel finding that transthyretin levels correlated with cognitive decline, the protein was assayed by a commercial ELISA kit (Assaypro-AssayMax Human prealbumin ELISA Kit). The assay was carried out as per the manufacturer's instruction. Baseline plasma samples from an independent cohort of AD subjects ($n = 270$) from both AddNeuroMed and KCL-ART were run in duplicate.

Genotyping

Venous blood was obtained for DNA extraction and genotyping for the apolipoprotein (APOE) alleles using standard methods [17]. The APOE haplotype was determined using two allelic discrimination assays (rs7412 and rs429358) based on fluorogenic 5′ nuclease activity: TaqMan single nucleotide polymorphism Genotyping Assays (Applied Biosystems).

Statistical analysis

Protein data was analyzed using SPSS version 17 (for Windows). Chi-square, student *t*-test, correlation analysis (Spearman non-parametric test) and non-parametric Mann-Whitney-Wilcoxon test were used to compare the sociodemographics, MMSE test scores and TTR protein levels between groups: rapid and non-rapid cognitive decliners; and mild and mod-severe AD subjects. Linear regression was performed with the loss of MMSE scores over 6 months follow up as the dependent variable and plasma transthyretin levels, age, baseline MMSE scores, duration of illness, gender, and APOE4 as predictive variables within the whole AD sample.

RESULTS

Discovery phase

We have previously reported the discovery phase experiments; comparing fast to slow progressors, using two-dimension gel electrophoresis (2DGE) and tandem mass spectrometry (LC/MS/MS) [15]. Proteins differing in plasma between fast and slow progressors included those previously identified by us, and by other groups, as potential markers for AD, including complement proteins and apolipoprotein A1. We have previously reported the validation studies for clusterin, a protein which is also altered in relation to degree of entorhinal cortex atrophy. One novel protein was identified in this discovery program, transthyretin (TTR), also known as pre-albumin.

Transthyretin levels lower in AD subjects compared to NDC

In order to validate this finding, we first compared the plasma TTR levels between AD subjects and age

Table 1
Comparison of plasma transthyretin level and socio-demographic-clinical parameters between the groups: a) AD subjects and non-demented controls; b) Within independent cohort of AD subjects: rapid cognitive decliners and non-rapid cognitive decliners and mild AD and moderate-severe AD

a) AD subjects and non-demented controls				b) Comparisons within an independent cohort of AD subjects ($n = 270$)					
Variables	AD ($n = 90$)	NDC ($n = 50$)	p-value	Rapid decliners ($n = 180$)	Non-rapid decliners ($n = 86$)	p-value	Mild AD ($n = 128$)	Mod-severe AD ($n = 142$)	p-value
Female/male	70/20	38/12	N.S[a]	120/60	57/29	N.S[a]	79/49	99/43	N.S[a]
Age, years	81.4 (6.5)	80.8 (7.2)	N.S[b]	77.5 (6.5)	77.1 (6.2)	N.S[b]	76.6 (5.9)	78 (6.6)	N.S[b]
TTR levels	63.9 (0.1)	82.3 (0.1)	0.01*¶	130.3 (64.6)	146.4 (62.9)	0.04*§	144.2 (61.2)	128.1 (65.8)	<0.01*§
APOE4	53 (60.2%)	9 (20%)	<0.001[a]	106 (60.6%)	46 (54.8%)	N.S[a]	71 (57.3%)	82 (60.5%)	N.S[a]
MMSE score, baseline	15.8 (8.1)	28.7 (1.1)	<0.001*	14.8 (7.6)	22.8 (4.7)	<0.001*	23.8 (2.6)	11.9 (6.5)	<0.001*
Duration of illness, months	n/a	n/a	n/a	3.9 (2.6)	6.3 (3.6)	<0.001[b]	4 (2.7)	5.2 (3.4)	<0.01[b]

Values are mean (SD) or n (%); [a]calculated using the χ^2 test, [b]calculated using the student t-test.
Wilcoxon paired test, ¶Western blotting experiments, §Enzyme linked immunoassay (ELISA).
AD, Alzheimer's disease; NDC, non-demented controls; MMSE, Mini Mental State Examination; TTR, transthyretin; APOE4, presence of one E4 allele; n/a, not applicable.

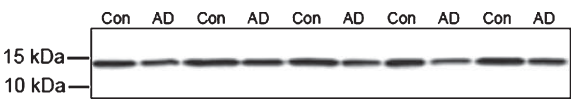

Fig. 1. Representative blot from immunoblotting experiment for plasma transthyretin levels in Alzheimer's disease patients and non-demented controls.

matched non-demented controls. Equal volumes of plasma from AD ($n = 90$) and controls ($n = 50$) (ART-KCL) were immunoblotted for TTR, in duplicate, as described above. A standard pooled sample was loaded in duplicate on each gel, to which each test sample was normalized, and which allowed inter-gel comparisons to be made. When assessing the reproducibility of the duplicate gels, a large positive correlation of 0.84 was obtained (Pearson correlation test). We found that TTR levels were significantly ($p = 0.004$) reduced in AD compared to NDC (Table 1a, Figs. 1 and 2). There was no association between APOE4 carrier status and TTR concentration ($p = 0.47$) as tested by analysis of covariance.

Transthyretin levels and cognition within AD subjects

We then used samples from an independent sample set of 270 AD subjects (AddNeuroMed = 177 and ART-KCL = 93). These included 178 females (66%), with a mean age of 77.4 years (±6.3) and mean MMSE score 17.49 (±7.8). APOE genotyping was available for 247 AD subjects, with 145 subjects having at least one E4 allele. The mean TTR level for the whole cohort was 135.5 μg/ml (±64). Information on anticholinesterase inhibitors treatment was available from the AddNeuroMed cohort, with $n = 123$ having

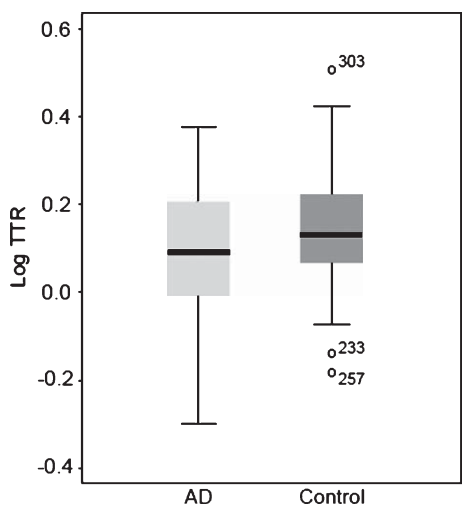

Fig. 2. Box plot showing lower plasma transthyretin levels in Alzheimer's disease subjects compared to non-demented controls.

ongoing treatment. We found no difference in the plasma TTR levels with and without treatment. There was also no difference in TTR levels between APOE4 carrier and non-carriers. There was no significant difference between depressed and non-depressed AD subjects as assessed by the neuropsychiatry inventory. There were 15 subjects with known thyroid dysfunction, however, there was no difference in TTR levels between subjects who had thyroid dysfunction ($n = 15$) and those without. There were no subjects with known liver dysfunction.

When comparing AD subjects by speed of decline, TTR levels were significantly lower in subjects with more rapid cognitive decline ($p = 0.036$), and also in subjects with moderate-severe AD ($p < 0.01$) (Table 1b) (Mann-Whitney U test).

Table 2
Linear regression analysis with the loss of MMSE scores over 6 months follow up as the dependent variable and plasma transthyretin levels, age, baseline MMSE scores, duration of illness, gender and APOE4 alternatively (Model 1) or simultaneously (Model 2) entered as predictive variables within the whole Alzheimer's disease sample

	R^2 (%)		Beta	T-value	P value
Model 1					
Plasma transthyretin	3.6		0.012	2.32	0.022*
Age in years	0.6		−0.039	−1.072	0.285
Duration of illness	0.4		−0.074	−0.924	0.356
MMSE baseline	1.8		0.092	1.903	0.058
Gender	0.2		−0.295	−0.592	0.555
APOE4	0.2		0.294	0.609	0.543
Model 2					
Plasma transthyretin + MMSE baseline	5.7	TTR	0.011	2.168	0.032*
		MMSE	0.100	1.779	0.077

R^2 (%) = R^2 value in percent for the overall model; *$p < 0.05$; MMSE, Mini Mental State Examination; TTR, Transthyretin; APOE4, presence of one E4 allele.

The change in MMSE scores from baseline over the following six months was then calculated. Linear regression analysis showed TTR levels as a better predictor factor for MMSE score change in the six months following venepuncture ($p = 0.029$), in both adjusted and unadjusted models with variables such as age, gender, duration of illness, baseline MMSE, and APOE4 carrier status (Table 2). Correlation analysis showed positive association of TTR levels with baseline MMSE scores; decreasing plasma TTR levels with lower MMSE scores ($p = 0.006$, $r^2 = 0.2$).

To determine the degree of correlation between the two techniques (ELISA and immunoblotting) used to measure TTR plasma levels, we performed both techniques on 40 new plasma samples, each run in duplicate in both assays. The samples were from 40 AD subjects (22 women) (AddneuroMed cohort), with a mean age 77.5 years (±6.6) and mean MMSE scores, 20.9 (±4.9). We found a good positive correlation between the two techniques ($p < 0.001$, $r^2 = 0.65$) (supplementary Figure 1; available online: http://www.j-alz.com/issues/28/vol28-2.html#supplementarydata06).

DISCUSSION

Previously we reported, in a discovery study, that TTR was one of the proteins in plasma discriminating between fast and slow progressing AD [15]. All other proteins from this discovery had been previously identified in biomarker studies [15]. Here we set out to determine whether this novel observation could be replicated in an independent sample set. By immunoblotting we found that TTR levels are significantly lower in AD subjects compared to the NDC. Measuring TTR by ELISA in an independent cohort of AD subjects, we found decreased TTR levels in moderate-severe stages of AD and in subjects presenting with rapid cognitive decline. We also found that plasma TTR level predicted subsequent decrease in MMSE score over the ensuing 6 months. The absolute concentration of TTR using TTR ELISA and immunoblotting correlated positively on a common set of plasma samples.

Previous studies have reported decreased TTR levels in CSF of patients with AD [18–23]. Low levels of TTR in CSF have been reported to be AD-specific compared with other dementia types, i.e., fronto-temporal dementia and Lewy body dementia [23, 24]. Lower TTR levels in CSF have been reported in severe AD [19, 22]. In a recent report, TTR is one of the six CSF biomarkers for AD describing six clinicopathological stages from cognitive normalcy to mild dementia, including stages defined by increased risk of cognitive decline [25]. Our findings are consistent with a recent report demonstrating lower serum TTR levels in AD subjects compared to NDC, although the study used a different detection method [26].

TTR, a 55-kDa homotetramer, is an abundant protein in CSF and human plasma, serving as the main transporter of thyroid hormones from the blood stream into CSF and in plasma, and is associated with retinol-binding protein [27]. It has been proposed that TTR acts as a scaffold protein, binding to Aβ and in so doing protects against Aβ deposition and the formation of senile plaques [28–30]. TTR seems to play an important role in keeping intracerebral proteins such as amyloid in a soluble form and helps prevent further aggregation [31]. In a recent study in mice, we found that deletion of insulin receptor substrate 2 (Irs2) resulting in insulin resistance increased tau pathology as expected but paradoxically decreased amyloid

pathology. We showed that this unexpected protection against plaque pathology was due to an increase in TTR expression [32], in line with a previous genome-wide expression study which found that increased TTR was one of the protective factors preventing transgenic mice with plaque pathology progressing to other pathological features of AD [33].

An alternative mechanism to explain the observation of lower TTR in more severe and more rapidly progressing AD is that TTR functions as a rate-limiting factor for the plasma transport of retinol [34]. Depletion of retinoic acid derivatives has been associated with deposition of Aβ peptides [35]. Whatever the mechanism, TTR is a prime candidate to influence Aβ pathology both directly and indirectly. The reasons for decreased plasma TTR levels in AD subjects could be from altered morphology of the choroid plexus in AD with possible change of expression profile including TTR production and its transport into blood [36]. Another possible explanation could be the down regulation of TTR expression in choroid plexus caused by activated β-secretase activity in AD with decreased sAβPPα [37]. Decreased hepatic TTR expression is another possible cause of reduced TTR in AD but none of our AD subjects had recorded liver dysfunction and additionally we did not find any differences in TTR levels of AD subjects with and without thyroid dysfunction.

In conclusion, significantly lower level of plasma TTR were found in AD subjects compared to non-demented controls and within AD subjects, TTR plasma levels were lower in subjects with rapid cognitive decline and severe cognitive impairment. In addition, TTR level predicted subsequent cognitive decline. These data suggest that plasma TTR is a strong candidate AD-specific biomarker that should be included in the development of blood-based biomarker panels for disease diagnosis and also suggests that plasma TTR may act as a marker for disease severity and progression.

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Authors' disclosures available online (http://www.j-alz.com/disclosures/view.php?id=999).

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CHAPTER 5

ENTORHINAL CORTEX THICKNESS AS A MARKER OF COGNITIVE DECLINE IN ALZHEIMER'S DISEASE

Paper 3

Entorhinal cortex thickness as a marker of cognitive decline in Alzheimer's disease

Velayudhan L, et al. Journal of Alzheimer's disease 2013; 33: 755-66

5.1 My contribution:

Another study related to the topic of Alzheimer's disease (AD) progression marker evolved during the thesis work whilst analysing the plasma proteins with neuroimaging done on the same patient cohort. I took lead in the data extraction, statistical analysis, interpretation, writing up the manuscript after detailed literature review and submission for publication. I then carried out necessary corrections following detailed and anonymous peer review process under the guidance of Dr Andy Simmons and my supervisors, Prof Simon Lovestone and Dr John Powell. I am the corresponding author for this paper.

5.2 Introduction

Biomarkers for AD based on non-invasive methods are highly desirable and neuroimaging markers provide an alternative and objective assessment of progression (Frank et al., 2003, Galasko, 2005).

A range of neuroimaging techniques provide insight into AD-related neurodegeneration, including structural magnetic resonance imaging (MRI), positron emission tomography (PET), and functional MRI. Neuroimaging techniques can improve early detection and aid in identifying individuals at risk of developing AD. In particular, structural MRI has provided insight into the neuroanatomical profile of pre-clinical and early AD. MRI has demonstrated significant value in the prediction of conversion and disease progression (Fennema-Notestine et al., 2009c).

From a neuropathological perspective, it has been suggested that the medial temporal lobe is the anatomical site of the first pathological alterations in Alzheimer's disease (Braak and Braak, 1991, Braak et al., 2006). It is well accepted that the medial temporal lobe plays an essential role in associative memory (Squire et al., 2004). It is clear that

the pathological processes of AD, which begin in the entorhinal cortex before spreading to hippocampus proper and other neocortical regions, affect the formation of new memories early in disease development. MRI is useful for detecting atrophy in the medial temporal structures affected early in the neurodegenerative process (Fennema-Notestine et al., 2009b). Decreased volumes of hippocampus and entorhinal cortex are connected to AD and that atrophy of the medial temporal lobe can predict conversion in subjects in the prodromal stages of the disease, MCI to AD (Fennema-Notestine et al., 2009a, Jack et al., 2004, Killiany et al., 2002, Varon et al., 2011).

The European Union AddNeuroMed multi-centre MRI study has reported that structural MRI measures discriminated AD from controls and MCI and also demonstrated potential for prediction of conversion from MCI to AD (Liu et al., 2010a, Westman et al., 2011c, Costafreda et al., 2011). The hippocampus, amygdala, and caudate volumes were significantly smaller in progressive MCI subjects than in controls and stable MCI subjects (Liu et al., 2010a). The volume of amygdala and caudate were independent variables in predicting conversion from MCI to AD in 100 amnestic MCI subjects, 118 AD patients, and 94 age-matched healthy controls from the AddNeuroMed study. The regional cortical thickness and volumes in MCI subjects were significantly decreased in limbic/paralimbic areas and temporal lobe compared to controls and also atrophy was much more extensive in the AD patients compared to MCI subjects and controls (Liu et al., 2011b). The use of whole-brain atrophy measured from serial MR imaging, correlates well with clinical progression (Fox et al., 1999, Sluimer et al., 2008). These studies have used baseline and serial MRI measures to predict future cognitive decline but mostly for conversion from MCI to AD and there is need for assessing these MRI measures as potential markers of disease progression in dementia due to AD.

The aims of the current study were to examine (a) the relationship between baseline hippocampal volume, entorhinal cortex thickness and whole brain volume with baseline cognitive measures in subjects with (i) AD dementia (ii) MCI and (iii) age matched non-demented controls (NDC); and (b) to assess the associations of the baseline MRI measures with subsequent cognitive change over one year period. The hypothesis was

that smaller brain structures would be associated with worse baseline cognition and greater cognitive decline.

5.3 METHODS

5.3.1 Subjects and assessments

This paper included data from 120 AD dementia, 106 mild cognitive impairment (MCI) and 99 non demented control (NDC) participants from the AddNeuroMed study, a European Union funded FP6 program (as described in Chapter 4, Subjects and samples) (Hye et al., 2006, Thambisetty et al., 2010, Velayudhan et al., 2012). Assessments included a structured interview including a detailed case and family history, Cambridge Examination for Mental Disorders of Older People (CAMDEX) (Roth et al., 1986); cognitive testing with Mini Mental State Examination (MMSE)(Folstein et al., 1975) and Alzheimer disease assessment scale – Cognitive (ADAS-cog) (Rosen et al., 1984) and stage of dementia with Clinical Dementia Rating (CDR) (Hughes et al., 1982) sum of boxes score. The cognitive testing with ADAS-cog and MMSE were repeated every 3 months for a period of a year. These subjects had structural MRI data which was acquired as designed to be compatible with the Alzheimer Disease Neuroimaging Initiative (ADNI) (Jack et al., 2008). This paper focussed on regional brain volumes and cortical thickness measures, specifically hippocampal volume, entorhinal cortex thickness and whole brain volume which have been proposed to be related to AD and have received high level of attention in the recent literature (Jack et al., 2004, Varon et al., 2011, Cardenas et al., 2011).

5.3.2 Statistical analysis:

Chi-square, non-parametric and T-test analyses were used to test for differences in MRI-based measures, cognition, severity measures, age, gender and education between AD dementia, MCI and NDC subjects. Correlation analysis (Spearman non-parametric test) was used for were used for associations between brain region volumes, cognitive scores (MMSE, ADAS-cog) and CDR for illness staging within the groups.

Rates of cognitive decline were determined by change in the cognitive measures – MMSE and ADAS-Cog total scores. These measures were estimated by fitting a random intercept and slope model using xtmixed in STATA 10 (Stata Corporation, College Station, TX, USA). The average baseline cognitive outcome and the average change in the cognitive outcome over the follow-up time were calculated for all the AD dementia patients, MCI and NDC as a group (fixed effects). Subject-specific intercept and slope terms which reflected deviation from the group average (random effects) were also calculated. Follow-up time was defined as the number of years (days/365.25) passed since the baseline visit, and up to 5 time points (three months apart) was recorded for each patient. Time squared was also used to assess nonlinear cognitive decline.

An interaction between the MRI-based brain volumes and follow- up time (Entorhinal Cortex X TIME, Whole Brain X TIME or Hippocampus X TIME) was used to test the null hypothesis that there was no difference in the rate of cognitive function i.e. in slopes for different baseline brain volumes. The coefficient of the time variable in this case (TIME) would indicate the association between follow-up time with cognitive decline for average brain volume (since the variables are centred around their mean); the coefficient of the brain volume for each subject (Entorhinal Cortex , Whole Brain or Hippocampus) would indicate the association of baseline brain volume with baseline cognitive assessment score and the coefficient of the interaction term (MRI brain volume x follow-up time) would indicate the effect of brain volume on cognitive decline over time. The results were based on using the brain measures as continuous variables and the quartiles for graphical view.

5.4 Discussion

The main findings of the study were (A) Patients with mild to moderate AD dementia had thinner ERC, smaller hippocampal volume and WBV compared to subjects with MCI and NDC. Within the AD group, (B) Baseline ERC and WBV were significantly associated with baseline cognition measured by MMSE, ADAS-cog and also with stage of dementia as measured by CDR sum of boxes scores. (C) Baseline ERC thickness but not hippocampal volume was associated with longitudinal changes in cognition over one year and could predict the degree of decline slopes as measured by MMSE and ADAS-

cog. (D) Baseline WBV was also associated with greater subsequent cognitive decline measured with ADAS-cog, although the association with the MMSE was marginal. The models were controlled for age at baseline, education years, gender, cholinesterase inhibitors, centre and apolipoprotein E genotype.

Pathologically, H. Braak and E. Braak demonstrated in post mortem brains that the hallmark neurofibrillary tangles evident in AD appear first in the prealpha transentorhinal neurons and then spread to the ERC proper (Braak and Braak, 1991). From here, the tangles spread to the subiculum (Sub) and Cornu Ammonis (CA) 1, then to CA 2 and 3 (Schonheit et al., 2004). The specificity of this pattern predicts specific cognitive deficits related to disease-related regionally specific neuronal death. This hypothesis was confirmed in a study which used high-resolution MRI, combined with a cortical unfolding technique to increase visibility of the convoluted medial temporal lobe, to assess whether grey matter thickness in subjects with MCI correlated to decline in cognition over two years, demonstrating that information in the initial encoding stage is most susceptible to early AD-related pathology and related to thinning in ERC and Sub (Burggren et al., 2011). In another study, atrophy rate of ERC was higher than that of hippocampus in patients with AD (n=20) evaluated twice approximately 1.9 years apart on volumetric T1-weighted MR images and the delayed list verbal recall test correlated significantly with atrophy rates ERC>hippocampus (Du et al., 2004). In a study wherein 71 non-demented participants were studied with structural MRI and neuropsychological testing at baseline and 1-year follow-up, smaller right hippocampal and entorhinal cortex (ERC) volumes at baseline were associated with worse delayed verbal memory performance at baseline while smaller left ERC volume was associated with greater longitudinal decline (Cardenas et al., 2011). Recently in a genome-wide study of atrophy in regions associated with neurodegeneration in AD, single-nucleotide polymorphism (SNP) with a disease-specific effect was associated with ERC volume in an intron of the ZNF292 gene (rs1925690) and an intergenic SNP, flanking the ARPP-21 gene, with an overall effect on entorhinal cortical thickness (rs11129640) (Furney et al., 2011). Gene-wide scoring also highlighted phosphatidylinositol-binding clathrin assembly protein (PICALM), one of the AD risk gene, as the most significant gene associated with entorhinal cortical thickness (Furney et al., 2011).

Developmental, morphological, functional and molecular features of layer II neurons in the ERC interact to promote early susceptibility of this cell type to aging and AD (Stranahan and Mattson, 2010). Within the ERC, there is subregional specificity for molecular alterations that may initiate cognitive decline and with a potential to directly contribute to downstream cascades in its primary afferent regions, the hippocampus (Stranahan and Mattson, 2010). Taken together, these findings suggest that AD is associated with progressive atrophy of both ERC and hippocampus, providing potential surrogate markers for this disease. Assuming that degenerative processes proceed at similar rates in the ERC and hippocampus, one might therefore expect to find higher atrophy rates in the structure where neuro-degeneration began earlier. Our study results substantiate this hypothesis, which is consistent with the view of earlier involvement of AD pathology in the ERC than the hippocampus (Braak and Braak, 1995).

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Entorhinal Cortex Thickness Predicts Cognitive Decline in Alzheimer's Disease

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Abstract. Biomarkers for Alzheimer's disease (AD) based on non-invasive methods are highly desirable for diagnosis, disease progression, and monitoring therapeutics. We aimed to study the use of hippocampal volume, entorhinal cortex (ERC) thickness, and whole brain volume (WBV) as predictors of cognitive change in patients with AD. 120 AD subjects, 106 mild cognitive impairment (MCI), and 99 non demented controls (NDC) from the multi-center pan-European AddNeuroMed study underwent MRI scanning at baseline and clinical evaluations at quarterly follow-up up to 1 year. The rate of cognitive decline was estimated using cognitive outcomes, Mini-Mental State Examination (MMSE) and Alzheimer disease assessment scale–cognitive (ADAS-cog) by fitting a random intercept and slope model. AD subjects had smaller ERC thickness and hippocampal and WBV volumes compared to MCI and NDC subjects. Within the AD group, ERC > WBV was significantly associated with baseline cognition (MMSE, ADAS-cog) and disease severity (Clinical Dementia Rating). Baseline ERC thickness was associated with both longitudinal MMSE and ADAS-cog score changes and WBV with ADAS-cog decline. These data indicate that AD subjects with thinner ERC had lower baseline cognitive scores, higher disease severity, and predicted greater subsequent cognitive decline at one year follow up. ERC is a region known to be affected early in the disease. Therefore, the rate of atrophy in this structure is expected to be higher since neurodegeneration begins earlier. Focusing on structural analyses that predict decline can identify those individuals at greatest risk for future cognitive loss. This may have potential for increasing the efficacy of early intervention.

Keywords: Alzheimer's disease, biomarker, cognitive decline, entorhinal cortex, hippocampus, whole brain volume

INTRODUCTION

Alzheimer's disease (AD) is the most common form of dementia and its prevalence is set to rise in the coming decades [1]. Biomarkers for AD, based on non-invasive methods are highly desirable for diagnosis, disease progression, and monitoring therapeutics

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[2, 3]. A range of neuroimaging techniques provide insight into AD-related neurodegeneration, including structural magnetic resonance imaging (MRI), positron emission tomography (PET), and functional MRI. Neuroimaging techniques can improve early detection and aid in identifying individuals at risk of developing AD. In particular, structural MRI has provided insight into the neuroanatomical profile of pre-clinical and early AD. MRI has demonstrated significant value in the prediction of conversion and disease progression [4].

From a neuropathological perspective, it has been suggested that the medial temporal lobe is the anatomical site of the first pathological alterations in AD [5, 6]. It has been shown that MRI is useful for detecting atrophy in the medial temporal structures affected early in the neurodegenerative process [4]. Decreased volumes of hippocampus and entorhinal cortex are connected to AD and to individuals at risk of developing the disease. It has been shown that atrophy of the medial temporal lobe can predict conversion in subjects in the prodromal stages of the disease, referred to as mild cognitive impairment (MCI) [4, 7–9]. Atrophy also correlates with memory impairment. Numerous studies have used baseline and serial MRI measures to predict future cognitive decline but mostly for conversion from MCI to AD [9–12], and there is need for assessing these MRI measures as potential markers of disease progression in AD.

We have previously reported from the European Union AddNeuroMed multi-center MRI study that structural MRI measures discriminated AD from controls and MCI; and also demonstrated potential for prediction of conversion from MCI to AD [13–18]. The aims of the current study were to examine (a) the relationship between baseline hippocampal volume, entorhinal cortex thickness, and whole brain volume with baseline cognitive measures in (i) AD (ii) MCI, and (iii) age matched non-demented controls (NDC); and (b) to assess the associations of the baseline MRI measures with subsequent cognitive change over one year period. Our hypothesis was that smaller brain structures would be associated with worse baseline cognition and greater cognitive decline.

METHODS

Participants and clinical assessment

This study included data from 120 AD, 106 MCI, and 99 NDC participants from the AddNeuroMed study, a European Union funded FP6 program. AddNeuroMed is a longitudinal, multi-center study of biomarkers for AD [19]. All subjects underwent MRI scanning at baseline and cognitive testing at baseline and every 3 months up to one year.

Data was collected from six different sites across Europe: University of Kuopio, Finland; University of Perugia, Italy; Aristotle University of Thessaloniki, Greece; King's College London, United Kingdom; University of Lodz, Poland; and University of Toulouse, France. Written consent was obtained where the research participant had capacity, and in those cases where dementia compromised capacity, then assent from the patient and written consent from a relative, according to local law and process, was obtained. This study was approved by ethical review boards in each participating country. The inclusion and exclusion criteria were as follows.

Alzheimer's disease

Inclusion criteria. Patients with probable mild to moderate AD (National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's disease and Related Disorders Association [NINCDS-ADRDA] criteria) [20] and Mini-Mental State Examination (MMSE) [21] score range between 12 and 28, age 65 years or above.

Exclusion criteria. Significant neurological or psychiatric illness, significant unstable systematic illness, or organ failure.

Mild cognitive impairment

Inclusion criteria. MMSE score range between 24 and 30, Clinical Dementia Rating (CDR) [22] scale score of 0.5, Geriatric Depression Scale score less than or equal to 5, age 65 years or above, medication stable, and good general health.

Exclusion criteria. Met the DSM- IV criteria for dementia, significant neurological or psychiatric illness, significant unstable systematic illness, or organ failure. The distinction between MCI and NDC was based on two criteria: CDR = 0 labeled the subject as control and a CDR = 0.5 labeled the subject as MCI. For the MCI subjects it was preferable that the subject and informant reported occurrence of memory problems.

Non-demented control

Inclusion criteria. MMSE score range between 24 and 30, CDR = 0, Geriatric Depression Scale score less

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than or equal to 5, age 65 years or above, medication stable, and good general health.

Exclusion criteria. Met the DSM- IV criteria for dementia, significant neurological or psychiatric illness, significant unstable systematic illness, or organ failure.

The clinical assessment and cognitive testing of the AddNeuroMed subjects followed a standard protocol described previously [13, 23, 24]. Assessments included a structured interview including a detailed case and family history, Cambridge Examination for Mental Disorders of Older People (CAMDEX) [25]; cognitive testing with MMSE and Alzheimer disease assessment scale – Cognitive (ADAS-cog) [26] and stage of dementia with CDR sum of boxes score. The cognitive testing with ADAS-cog and MMSE were repeated every 3 months for a period of a year.

Genotyping

Venous blood was obtained for DNA extraction and genotyping for the apolipoprotein (APOE) alleles using standard methods [27]. The APOE haplotype (rs7412 and rs429358) was determined using two allelic discrimination assays based on fluorogenic 5′ nuclease activity: TaqMan single nucleotide polymorphism Genotyping Assays (Applied Biosystems,).

Magnetic resonance imaging

Data acquisition for the AddNeuroMed study was designed to be compatible with the Alzheimer Disease Neuroimaging Initiative (ADNI) [28]. The imaging protocol included a high resolution sagittal 3D T1-weighted MPRAGE volume (voxel size $1.1 \times 1.1 \times 1.2\,\mathrm{mm}^3$) and axial proton density/T2-weighted fast spin echo images. The MPRAGE volume was acquired using a custom pulse sequence specifically designed for the ADNI study to ensure compatibility across scanners [28]. Full brain and skull coverage was required and detailed quality control was carried out on all MR images according to the AddNeuroMed quality control procedure [23, 29].

Regional volume segmentation

We applied the Freesurfer pipeline (version 4.5.0) to the MRI images to produce regional cortical thickness and subcortical volumetric measures. Cortical reconstruction and subcortical volumetric segmentation includes removal of non-brain tissue using a hybrid watershed/surface deformation procedure [30], automated Talairach transformation, segmentation of the subcortical white matter and deep grey matter volumetric structures (including hippocampus, amygdala, caudate, putamen, ventricles) [30–32], intensity normalization [33], tessellation of the grey matter white matter boundary, automated topology correction [34, 35], and surface deformation following intensity gradients to optimally place the grey/white and grey/cerebrospinal fluid borders at the location where the greatest shift in intensity defines the transition to the other tissue class [36–38]. Once the cortical models are complete, registration to a spherical atlas takes place which utilizes individual cortical folding patterns to match cortical geometry across subjects [39]. This is followed by parcellation of the cerebral cortex into units based on gyral and sulcal structure [40, 41]. This segmentation approach has been used for multivariate classification of AD and healthy controls [16, 17, 42, 72], neuropsychological-image analysis [15, 18], imaging-genetic analysis [43, 44], and biomarker discovery [24, 73, 74]. The current study focused on regional brain volumes and cortical thickness measures, specifically hippocampal volume, entorhinal cortex (ERC) thickness, and whole brain volume (WBV) which have been proposed to be related to AD and have received high level of attention in the recent literature [4, 7–12, 16, 17]. Volumes from the left and the right hemisphere were averaged together. All volumetric measures from each subject were normalized by the subject's intracranial volume. Cortical thickness measures were not normalized and were used in their raw form [45].

Statistical analysis

Non-parametric and *t*-test analyses were used to test for differences in continuous outcomes such as MRI-based measures, cognition, severity measures, age, and education between AD, MCI, and NDC. The chi-square test was used to test for differences in categorical outcomes such as gender and the presence of the APOE $\varepsilon 4$ allele. Correlation analysis (Spearman non-parametric test) was used for associations between brain region volumes, cognitive scores (MMSE, ADAS-cog), and CDR for illness staging within the groups.

Rates of cognitive decline were determined by change in the cognitive measures (MMSE and ADAS-Cog total scores). These measures were estimated by fitting a random intercept and slope model using xtmixed in STATA 10 (Stata Corporation, College

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Station, TX, USA). The average baseline cognitive outcome and the average change in the cognitive outcome over the follow-up time were calculated for all the AD patients, MCI, and NDC as a group (fixed effects). Subject-specific intercept and slope terms which reflected deviation from the group average (random effects) were also calculated. Follow-up time was defined as the number of years (days/365.25) passed since the baseline visit, and up to 5 time points (three months apart) was recorded for each patient. Time squared was also used to assess nonlinear cognitive decline.

Adjustment for age at baseline, education years, gender, cholinesterase inhibitors, center, and APOE genotype was made. Continuous outcomes were centered to their mean to aid interpretation of the model. As the main focus of the study was to study associations for cognitive decline in AD, we did not differentiate MCI into converters and non-converters and used all MCI as a group.

An interaction between the MRI-based brain volumes and follow- up time (Entorhinal Cortex × TIME, Whole Brain × TIME, or Hippocampus × TIME) was used to test the null hypothesis that there was no difference in the rate of cognitive function, i.e., in slopes for different baseline brain volumes. The coefficient of the time variable in this case (TIME) would indicate the association between follow-up time with cognitive decline for average brain volume (since the variables are centered around their mean); the coefficient of the brain volume for each subject (Entorhinal Cortex, Whole Brain, or Hippocampus) would indicate the association of baseline brain volume with baseline cognitive assessment score and the coefficient of the interaction term (MRI brain volume × follow-up time) would indicate the effect of brain volume on cognitive decline over time. The results were based on using the brain measures as continuous variables and the quartiles for graphical view.

RESULTS

Demographics, brain region, and baseline cognition

The subject characteristics are shown in Table 1. Predictably the AD patients had smaller regional brain measures and lower cognitive scores compared to age-matched MCI and NDC subjects. Within AD subjects, ERC volumes correlated significantly with baseline MMSE ($p < 0.01$, $r^2 = 0.3$), ADAS-cog ($p < 0.01$, $r^2 = -0.3$), and CDR scores ($p < 0.001$, $r^2 = -0.3$) and WBV with CDR ($p < 0.001$, $r^2 = -0.3$). Within NDC, MMSE correlated with WBV ($p = 0.02$, $r^2 = -0.2$) and with hippocampal volume ($p = 0.04$, $r^2 = -0.2$). Within the MCI group, there were no significant correlations between MMSE and brain volumes.

Brain region and longitudinal changes in cognition

We did not identify any deviation from a linear cognitive decline model by including the TIME squared variable in the model (non-significant coefficient) and all the models therefore assumed a linear cognitive decline.

Association of baseline ERC thickness with cognitive decline in AD subjects

Mixed effects models indicated a significant interaction between follow-up time measured with the

Table 1
Demographics and brain volumes between subjects with Alzheimer's disease (AD), mild cognitive impairment (MCI), and non-demented controls (NDC)

	AD ($n = 120$)	MCI ($n = 106$)	NDC ($n = 99$)	$p < 0.001$**	$p < 0.05$**
Gender (Female %)	64	49	53	NS¶	a
Age in years	74.82 (6.21)	74.00 (5.64)	74.56 (5.14)	NS*	
Education	7.91 (4.01)	9.03 (4.18)	10.67 (4.89)	b*	a, c
APOE4 (%)	56	38	28	b¶	a
MMSE	20.83 (4.83)	27.21 (1.64)	28.96 (1.28)	a, b, c*	
Hippocampus (cm^3)	1.95 (0.37)	2.27 (0.35)	2.67 (0.27)	a, b, c*	
% reduction to NDC	27	15			
Entorhinal cortex (mm)	2.62 (0.53)	3.00 (0.36)	3.24 (0.36)	a, b, c*	
% reduction to NDC	19	7			
Whole brain volume	0.82 (0.04)	0.85 (0.03)	0.85 (0.03)	a, b*	
% reduction to NDC	4	0			

Mean (SD); ¶, chi-square; *, *t*-test; MMSE, Mini-Mental State Examination; APOE4, presence of at least one e4 allele. **Multiple comparisons abbreviated as: (a) AD subjects differ from subjects with MCI, (b) AD subjects differ from NDC subjects, (c) MCI subjects differ from NDC subjects.

Table 2
Mixed effects regression for subjects with Alzheimer's disease (AD), non-demented control (NDC), and mild cognitive impairment (MCI) over one year, adjusted for age, gender, center, education, APOE ε4, and cholinesterase inhibitor therapy in AD group

Baseline brain area	Variable	AD ($n = 120$)						NDC ($n = 99$)			MCI ($n = 106$)		
		MMSE			ADAS-cog			MMSE			MMSE		
		Beta	SE	p	Beta	SE	p	Beta	SE	p	Beta	SE	p
Entorhinal cortex	Time (years)	–1.333	0.334	<0.001	2.675	0.664	<0.001	–2.020	1.135	0.075	–0.694	0.296	0.019
	Entorhinal cortex thickness	2.661	0.755	<0.001	–5.083	1.555	0.001	0.157	0.361	0.665	–0.036	0.341	0.916
	Time (years) × ERC thickness	1.705	0.648	0.009	–5.737	1.282	<0.001	0.002	0.001	0.094	0.234	0.586	0.689
Whole brain volume	Time (years)	–1.300	0.331	0.000	2.505	0.696	0.000	–2.112	3.502	0.546	–0.726	0.287	0.012
	Whole brain volume	0.029	0.012	0.016	–0.065	0.024	0.006	2.263	4.947	0.647	3.614	5.883	0.539
	Time (years) × WB volume	0.018	0.009	0.049	–0.052	0.019	0.007	0.006	0.011	0.575	–0.197	8.471	0.981
Hippocampus	Time (years)	–1.322	0.336	<0.001	2.574	0.710	<0.001	0.942	1.245	0.449	–0.633	0.287	0.028
	Hippocampal volume	2.471	1.105	0.025	–2.471	2.255	0.273	0.455	0.219	0.038	0.468	0.531	0.378
	Time (years) × Hippo volume	0.219	0.906	0.809	–2.509	1.912	0.189	–0.001	0.001	0.391	0.015	0.854	0.986

Coefficients of the interaction terms (brain measure × time) represented the influence of baseline brain measures on rates of change. Time (years) represents the association of follow-up time with cognitive decline for mean brain measures, and the respective brain measure coefficients represent the association of baseline measures with cognitive decline at baseline. MMSE, Mini-Mental State Examination; ADAS-cog, Alzheimer disease assessment scale–Cognitive.

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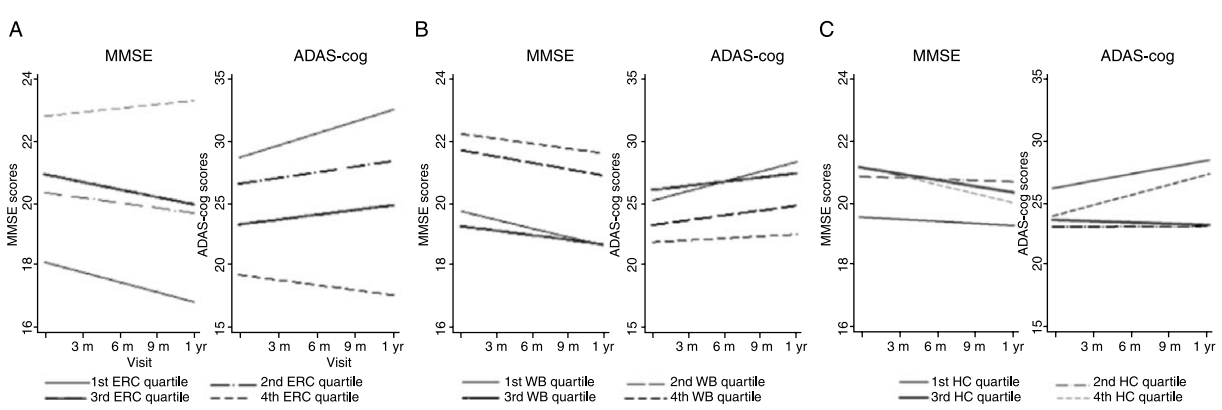

Fig. 1. Mini-Mental State Examination (MMSE) and Alzheimer disease assessment scale–Cognitive (ADAS-cog) decline over the quarterly visits for a year for Alzheimer's disease subjects in the four entorhinal cortex (ERC), whole brain volume (WBV), and hippocampus (HC) quartiles. MMSE score represents number of correct items; ADAS-cog score represent the number of errors. A) ERC thickness in mm [mean (SD)]: 1st ERC quartile: 1.97 (0.21); 2nd ERC quartile: 2.42 (0.10); 3rd ERC quartile: 2.83 (0.11); 4th ERC quartile: 3.31 (0.26). B) WBV divided by intracranial volume [mean (SD)]: 1st WBV quartile: 0.77 (0.02); 2nd WBV quartile: 0.81 (0.01); 3rd WBV quartile: 0.84 (0.01); 4th WBV quartile: 0.87 (0.02). C) HC volume in cm^3 [mean (SD)] 1st HC quartile: 1.47 (0.17); 2nd HC quartile: 1.83 (0.09); 3rd HC quartile: 2.08 (0.06); 4th HC quartile: 2.40 (0.19).

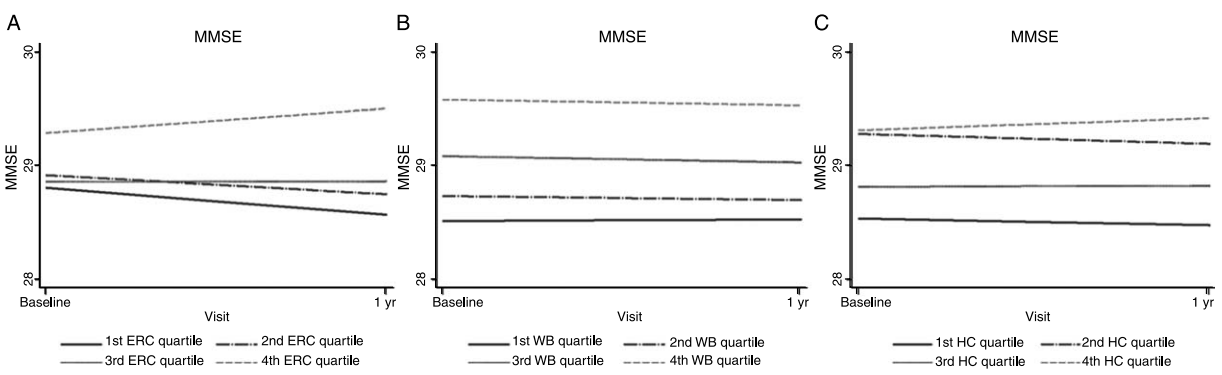

Fig. 2. Mini-Mental State Examination (MMSE) decline over 1 year for non-demented control subjects in the four entorhinal cortex (ERC), whole brain volume (WBV), and hippocampus (HC) quartiles showing no significant associations. MMSE score represents number of correct items. A) ERC thickness in mm [mean (SD)]: 1st ERC quartile: 2.76 (0.20); 2nd ERC quartile: 3.15 (0.07); 3rd ERC quartile: 3.37 (0.06); 4th ERC quartile: 3.67 (0.18). B) WBV divided by intracranial volume [mean (SD)]: 1st WBV quartile: 0.81 (0.02); 2nd WBV quartile: 0.85 (0.00); 3rd WBV quartile: 0.86 (0.01); 4th WBV quartile: 0.89 (0.01). C) HC volume in cm^3 [mean (SD)]: 1st HC quartile: 2.31 (0.12); 2nd HC quartile: 2.58 (0.06); 3rd HC quartile: 2.78 (0.06); 4th HC quartile: 3.01 (0.12).

MMSE and ADAS-Cog and baseline ERC thickness ($p = 0.009$ and $p < 0.001$, respectively) which indicated that baseline ERC thickness was related to the rate of cognitive decline measured with these two cognitive scales (Table 2).

Higher baseline ERC thickness was associated with slower cognitive decline measured with MMSE and ADAS-cog. In more detail, after adjusting for covariates, higher baseline ERC thickness in AD cases were associated with both higher baseline cognition measured with the MMSE and ADAS-cog measures (beta = 2.661 (0.755), $p < 0.001$ and beta = −5.083 (1.55), $p = 0.001$, respectively) and with slower cognitive decline, measured with MMSE (beta = 1.705 (0.648), $p = 0.009$) and ADAS-cog (beta = −5.737 (1.282), $p < 0.001$). To aid the interpretation, Fig. 1A displays the predicted MMSE and ADAS-cog slopes for the four baseline ERC thickness quartiles, highlighting the differences both in baseline cognitive scores but also in the rate of cognitive decline between different ERC quartiles, especially, for patients in the 4th quartile. For example, the expected average MMSE decline for patients in the lower ERC quartile was −2.34 per year ($p = 0.001$), whereas there was no

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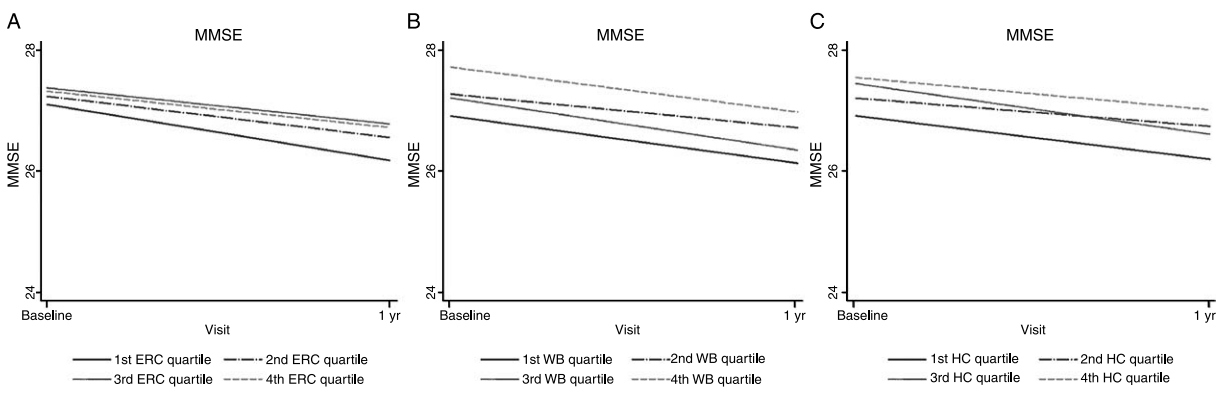

Fig. 3. Mini-Mental State Examination (MMSE) decline over 1 year for mild cognitive impairment subjects in the four entorhinal cortex (ERC), whole brain volume (WBV), and hippocampus (HC) quartiles showing no significant associations. MMSE score represents number of correct items. A) ERC thickness in mm [mean (SD)]: 1st ERC quartile: 2.30 (0.27); 2nd ERC quartile: 2.85 (0.14); 3rd ERC quartile: 3.25 (0.11); 4th ERC quartile: 3.62 (0.19). B) WBV divided by intracranial volume [mean (SD)]: 1st WBV quartile: 0.80 (0.02); 2nd WBV quartile: 0.84 (0.01); 3rd WBV quartile: 0.8 (0.01); 4th WBV quartile: 0.89 (0.01). C) HC volume in cm^3 [mean (SD)]: 1st HC quartile: 1.86 (0.18); 2nd HC quartile: 2.18 (0.05); 3rd HC quartile: 2.39 (0.07); 4th HC quartile: 2.72 (0.18).

significant decline for patients in the upper ERC quartile (beta = 0.372, $p = 0.557$). The same effect was observed for ADAS-Cog.

Association of baseline WBV with cognitive decline in AD subjects

As in the case of ERC, mixed effect models indicated that baseline WBV was associated with higher baseline cognitive scores (MMSE beta = 0.028 (0.012), $p = 0.016$; ADAS-cog beta = −0.065 (0.024), $p = 0.006$) and also appeared to modify the rate of cognitive decline measured with MMSE and ADAS-cog, although the effect on MMSE measured decline was only marginal (Table 2). In more detail, baseline WBV appeared to have a strong influence on the rate of cognitive decline measured with ADAS-cog (beta = −0.052 (0.019), $p = 0.007$) and showed a modest effect on MMSE assessed decline (beta = 0.018 (0.009), $p = 0.049$). Lower baseline WBV predicted cognitive decline when assessed with the ADAS-cog and also to an extent with the MMSE (Fig. 1B, Table 2).

Association of baseline hippocampal volume with cognitive decline in AD subjects

Finally, mixed effects models indicated that the baseline volume of the hippocampus was associated with baseline MMSE (beta = 2.471 (1.105), $p = 0.025$), i.e., patients with larger hippocampus volumes had higher MMSE (Table 2), but was not associated with baseline ADAS-cog scores, neither did it seem to modify the rate of cognitive decline assessed by the two cognitive tools (Fig. 1C, Table 2).

Association of baseline ERC, WBV, and hippocampal volume with cognitive decline in NDC and MCI subjects

Finally, mixed effects models indicated that the baseline ERC thickness, WBV, and volume of the hippocampus was not associated with cognitive decline measured using MMSE in control and MCI subjects, over a period of one year (Table 2, Figs. 2 and 3).

DISCUSSION

The main findings of the study were: (A) patients with mild to moderate AD had thinner ERC, smaller hippocampal volume, and WBV compared to subjects with MCI and NDC. Within the AD group, (B) baseline ERC and WBV were significantly associated with baseline cognition measured by MMSE and ADAS-cog and also with stage of dementia as measured by CDR sum of boxes scores. (C) Baseline ERC thickness but not hippocampal volume was associated with longitudinal changes in cognition over one year and could predict the degree of decline slopes as measured by MMSE and ADAS-cog. (D) Baseline WBV was also associated with greater subsequent cognitive decline measured with ADAS-cog, although the association with the MMSE was marginal. The models were controlled for age at baseline, education years, gender, cholinesterase inhibitors, center, and APOE genotype.

Reductions in the hippocampal and entorhinal regions between the AD and NDC in our study were similar to previous studies [4, 46]. The differences in these regions between MCI and NDC were also

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comparable with a previous study [46]. We found greater reductions in hippocampus compared to ERC regions similar to these studies in both AD and MCI groups. However, the focuses of these studies were on predictors of MCI conversion to AD, and they did not report on the association between the regions and the cognitive measures within the AD group.

Our findings are in line with previous studies that ERC measures correlate better with baseline cognitive scores than hippocampal volume and that atrophy in ERC predicts cognitive decline better than hippocampal atrophy [7, 11, 47–48]. Recently in a genome-wide study of atrophy in regions associated with neurodegeneration in AD, we identified one single-nucleotide polymorphism (SNP) with a disease-specific effect associated with ERC volume in an intron of the ZNF292 gene (rs1925690) and an intergenic SNP, flanking the ARPP-21 gene, with an overall effect on entorhinal cortical thickness (rs11129640) [41]. Gene-wide scoring also highlighted PICALM as the most significant gene associated with ERC thickness [49].

Although we found that hippocampal volume associated with the baseline cognitive measures as previously reported [8, 50, 51], we found no association between hippocampal volume and subsequent cognitive change, which has also been previously reported by some [52, 53], but not all authors [54]. Mungas and colleagues previously reported that hippocampal atrophy predicted decline in AD but only in those subjects without lacunes [12].

WBV correlated with baseline clinical measures and predicted future cognitive decline, which probably reflects the correspondence between these measures of overall cerebral loss and global cognitive measures in the moderate stages of AD as reported earlier [52, 55].

Structures within the temporal lobe have long been associated with AD decline because of their critical role in the formation of long-term memory, one of the first functions to be affected in disease progression [51]. Both ERC and hippocampus are essential parts of the medial temporal lobe system that supports declarative (conscious) memory [56]. AD pathology primarily begins in ERC, followed by immediate progression through subiculum to the hippocampus proper [5]. Pathologically, Braak and Braak demonstrated that the spread of neurofibrillary tangles in postmortem brains appear first in the prealpha transentorhinal neurons and then spread to the ERC proper [5]. Developmental, morphological, functional, and molecular features of layer II neurons in the ERC interact to promote early susceptibility of this cell type to aging and AD [57]. Within the ERC, there is subregional specificity for molecular alterations that may initiate cognitive decline and with a potential to directly contribute to downstream cascades in its primary afferent regions, the hippocampus [57]. Previous clinical studies demonstrated that the rates of cognitive decline accelerated with time in AD [58, 59], suggesting accelerated neurodegeneration in AD. Both cross-sectional and serial MRI studies on patients with AD have consistently found volume losses in both ERC and hippocampus [48, 51, 60–64]. Taken together, these findings suggest that AD is associated with progressive atrophy of both ERC and hippocampus, providing potential surrogate markers for this disease. Assuming that degenerative processes proceed at similar rates in the ERC and hippocampus, one might therefore expect to find higher atrophy rates in the structure where neurodegeneration began earlier. Our results substantiate this hypothesis, which is consistent with the view of earlier involvement of AD pathology in the ERC than the hippocampus [65]. Risacher et al., however, have found similar atrophy rates of 4-5% per year in the hippocampus and entorhinal cortex in the ADNI cohort [66].

APOE ε4 had no influence on the relation between the ERC, hippocampal, and WBV with cognitive severity or cognitive decline in our study, similar to other reports [67, 68]. We have previously reported that the homozygous ε4 carriers had significant volume loss in hippocampus and amygdala in AD [43]. Possibly the influence is not seen due to lack of association of hippocampus volume with cognitive severity or longitudinal cognitive decline in AD. This warrants further evaluation in future longitudinal studies. We found significant correlations between structural MRI measures and baseline MMSE for healthy control subjects in our study, but the small range of MMSE for healthy subjects means that this finding should be viewed with some caution.

Strengths of the current study include the sample size and our use of automated MRI measures. The study compares cognitive decline for AD, MCI, and NDC subjects using the baseline brain measures as continuous variables and the quartiles for graphical view.

In conclusion, subjects with AD had thinner ERC, smaller WBV, and hippocampal volume compared to subjects with MCI and NDC. In addition, with in AD subjects, thinner ERC was associated with lower baseline cognitive scores, higher disease severity, and predicted greater subsequent cognitive decline at one year follow up. MRI is superior in defining disease stage clinically and has been shown to be a slightly

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better predictor of future clinical decline than cerebrospinal fluid biomarkers [69, 70]. Neuroimaging biomarkers that predict decline would have a great potential for increasing the efficacy of early intervention [71]. By focusing structural analyses on regions known to be first affected in AD, we may better identify those individuals at greatest risk for future memory decline, valuable in determining the course of future care needed by these individuals, requiring more substantial care at an earlier time point.

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CHAPTER 6

GENERAL DISCUSSION AND FUTURE DIRECTIONS

DISCUSSION

6.1 SUMMARY OF KEY FINDINGS:

The aim of the thesis was to investigate markers of progression in AD dementia, using different strategies – clinical and laboratory based. Firstly, I carried out a longitudinal clinical study to evaluate the utility of olfaction dysfunction as marker of AD disease progression. To the best of my knowledge, this is the first study to report smell identification test as a marker of severity in mild-moderate AD (Velayudhan et al., 2013a), although previously been studied as a diagnostic marker by various groups.

In parallel, I tested plasma proteins identified from a discovery study discriminating fast and slow progressing AD dementia and the published paper (Thambisetty et al., 2010) included in the thesis was the first study using multiple experiments in human and animal models to demonstrate the association of protein clusterin with AD pathology, severity and disease progression.

Transthyretin (TTR) was one of the plasma proteins discovered in the gel proteomics discovery discriminating fast and slow progressing AD dementia (Chapter 3, a). All other proteins from this discovery phase had been previously identified in biomarker studies (Thambisetty et al., 2010). I set out to determine whether the novel observation could be replicated and demonstrated it to be a marker of severity and cognitive decline in an independent and larger patient cohort (Velayudhan et al., 2012). Again, there are no previous studies examining this.

Lastly, I examined structural MRI brain regions to predict future cognitive decline. The third paper (Velayudhan et al., 2013b), which showed entorhinal cortex thickness predicted cognitive decline in moderate AD dementia, is in line with previous studies. However, this paper compares prospective cognitive decline for AD dementia, mild cognitive impairment and normal control subjects, using the baseline brain measures as continuous variables and the quartiles for graphical view.

6.2 DISEASE PROGRESSION IN AD

Disease progression is measured, most commonly, by change in cognition over time (Kraemer et al., 1994, Marra et al., 2000, Schmidt et al., 2011, Behl et al., 2005). The studies in the thesis defined rapid progression based on the rates of cognitive decline at the time of presentation and also tested the progression markers for associations with prospective cognitive decline. It is interesting to note that these markers which associated with disease severity at time of presentation and the initial rates of decline also predicted the subsequent progression. This is in keeping with observations from previous studies that an estimate of initial rates of decline at the time of presentation ('how far') would be predictive of subsequent disease progression ('how fast'), i.e., initial rapid progressors will continue to decline faster than the initial slow progressors (Kraemer et al., 1994, Doody et al., 2001).

Annualised rate of change in MMSE was used to define the rapid and slow decliners (olfaction and plasma marker studies). Studies report annual rates of change (ARC) of mental status evaluations, as one way of gauging disease progression that is more specific and less variable as a measure of disease deterioration than clinical endpoints (Behl et al., 2005, Galasko et al., 2000). This calculation assumes linear change over the time intervals, which may be stage dependent and may not be true for long intervals (Behl et al., 2005). I also used statistical approaches, such as, regression by least squares, for prospective decline, which has been used to address nonlinear change (Galasko et al., 2000).

Interestingly all the progression markers also indicated severity of illness- TTR and Clusterin levels for cognitive severity with MMSE; olfaction impairment for lower MMSE, functional dependence and more neuropsychiatry symptoms; and thinner ERC and smaller whole brain volume for CDR sum of boxes. Previous studies have found such scales providing an index of the severity of the neuropathology of AD, such as, a significant correlation between MMSE scores and neurofibrillary tangles within the Nucleus Basalis of Meynert (Iraizoz et al., 1999, Behl et al., 2005) .

6.3 Effects of APOE4 on the markers of AD progression:

APOE4 status had no effect on the concentrations of plasma TTR, plasma clusterin or different brain region volumes for their influence on the disease progression. The disease progressions in all these studies were measured as cognitive decline. The association of APOE with cognitive decline in the literature vary. Several studies report a positive association of APOE ε4 with more rapid cognitive decline (Corder et al., 1993, Martins et al., 2005, Craft et al., 1998, O'Hara et al., 2008). Conversely, in an individual growth curve analysis of APOE ε4-associated cognitive decline in AD, report that although the APOE ε4allele is associated with an increased risk of developing AD, subjects with 2 ε4 alleles have a slower clinical course (Hoyt et al., 2005). However, there is also report that although APOE ε4 increased the risk for AD and decreased the age of disease onset in population studies, it did not significantly influence the rate of disease progression in cognitive or functional domains (Kleiman et al., 2006). The results from thesis studies (paper 1, 2 and 3) which were all derived from AddNeuromed multi-centred cohort are in keeping with lack of association of the APOE4 status with markers for cognitive decline (Schmidt et al., 2010, Schmidt et al., 2011, Wilkosz et al., 2010). Olfaction study which examined a different clinical population from 2 UK centres however did not test for the APOE4 status and this is something to look at in future studies.

6.4 FURTHER DISCUSSIONS AND DEVELOPMENT ON PLASMA MARKERS SINCE PUBLICATION

6.4.1 CLUSTERIN

Single-nucleotide polymorphisms in the clusterin gene, *CLU*, were recently found to be associated with the risk of developing late-onset AD (Harold et al., 2009, Lambert et al., 2009). Clusterin, a multifunctional lipoprotein, comprises disulphide linked _ (34–36 kDa) and (~36–39 kDa) peptides (de Silva et al., 1990). It is expressed in a number of tissues, but expression is particularly high in the brain (de Silva et al., 1990, Nuutinen et al., 2009).

Although the work to date has not confirmed the clinical utility of plasma clusterin concentration as a stand-alone biomarker for AD, this study establishes this amyloid chaperone protein as a robust peripheral signature that is responsive to key features of disease pathology. The research clearly implicates clusterin but there may well be other proteins in plasma related to disease process. Nevertheless, this data has attracted major collaborative translational funding for long term trials and biomarker validation. Further work done has been done by the research group (Thambisetty et al., 2012b, Thambisetty et al., 2012a, Hardy et al., 2011)and replicated by other groups since our data publication (Schrijvers et al., 2011). In a recent study the AD genetic risk variant rs11136000 in *CLU* is found to influence longitudinal changes in brain function in asymptomatic individuals and is associated with faster cognitive decline in presymptomatic stages of disease progression, suggesting mechanisms underlying the role of *CLU* in AD and important in monitoring disease progression in at-risk elderly (Thambisetty et al., 2012b). Plasma clusterin was found to be associated with rate of brain atrophy in MCI and reflect its concentration within brain regions vulnerable to AD pathology (Thambisetty et al., 2012a). Another study in a cohort of 3709 participants found plasma clusterin levels significantly associated with baseline prevalence and severity of AD measured by the MMSE, but the study did not examine association with disease progression in AD (Schrijvers et al., 2011). In a recent study the association of the AD clusterin common risk polymorphism rs9331888 with blood clusterin levels was tested in 104 AD subjects and 104 healthy controls (Xing et al., 2012). Blood clusterin levels were significantly elevated in AD patients compared to control subjects, however, the rs9331888 AD-risk variant was associated with low clusterin mRNA and protein levels in an allele-dose dependent manner in both groups.

The many roles of clusterin make it a candidate neuroprotective agent and the neurodegenerative changes that occur in AD may trigger an increased expression of clusterin (Nuutinen et al., 2009). Several protective effects of clusterin on the brain that may play a role in AD have been described in in vitro or in vivo studies, including inhibition of amyloid formation (Yerbury et al., 2007) through binding amyloid-beta or enhancing its clearance over the blood-brain barrier (Bell et al., 2007), clearance by endocytosis of amyloid beta aggregates and cell debris to brain phagocytes, involved in

regulation of brain cholesterol and lipid metabolism, inflammation of the brain, and the inhibition of neuronal apoptosis/ potentiation of neuroprotection (Nuutinen et al., 2009). Thus, clusterin is not a typical target, since it has so many functions and isoform-specific effect; however, this may be an advantage for a multi-etiological disorder such as AD (Yu and Tan, 2012).

6.4.2 TRANSTHYRETIN

Transthyretin (TTR) is a homotetrameric protein of 55KDa found mainly in plasma and cerebrospinal fluid. In 1981, the International Union of Biochemists adopted the name ' transthyretin' to change the prior descriptive term- 'thyroxine binding prealbumin' to more preferable functional nomenclature based on its physiologic role (*trans*porter of *thy*roxine and *retin*ol) (Buxbaum and Reixach, 2009).

A role of TTR, in association with retinol-binding protein, is to transport retinol (vit A) from liver storage to target tissues where it can be metabolized to retinoic acid in response to physiologic needs (Monaco, 2000). Some evidence has suggested a role of perturbed RA pathway in age related memory impairments (Chiang et al., 1998, Etchamendy et al., 2001, Misner et al., 2001, Cocco et al., 2002). Knock-out mice lacking TTR showed spatial memory deficits during aging (Brouillette and Quirion, 2008). They also demonstrated reversal of cognitive deficits with R treatment in $TTR^{-/-}$ mice and aged rats suggesting that the mechanisms underlying the role of TTR in memory formation during aging is linked to its ability to regulate brain retinoid availability. Taken together, these results provide evidence that TTR is involved in the maintenance of memory capacities during aging.

The formation of insoluble aggregates of amyloid-β peptides (Aβ) in the brain is considered critical event in AD (Hardy and Selkoe, 2002). Aβ, consisting of 39-43 amino acids, is produced from amyloid- β protein precursors (AβPP) by the combined actions of β and γ secretase (Kang et al., 1987). Among the Aβ species, the Aβ1-42 peptide has the greatest tendency to form amyloid fibrils (Barrow and Zagorski, 1991) and it is the peptide initially deposited in the plaques (Miller et al., 1993). An alternative non-

amyloidogenic cleavage of AβPP by a α-secretase generates soluble α-sAβPP, a fragment that might be neuroprotective (Goodman and Mattson, 1994, Thornton et al., 2006).

In vitro studies have suggested TTR can bind to Aβ1-40 and Aβ1-42 monomers, preventing its transformation into toxic fibrils and amyloid plaques (Schwarzman et al., 1994, Buxbaum et al., 2008, Liu and Murphy, 2006). TTR seems to play an important role in keeping intracerebral proteins such as amyloid in a soluble form and helps prevent further aggregation (Liu and Murphy, 2006). Binding of the aggregates are better than that of the monomers and it also appears that the affinity of TTR is greater for Aβ1-42 than for Aβ1-40.

Earlier studies in Tg2576 AD model mice showed that *ttr* transcripts were increased and TTR protein was immunochemically detected in neurons in hippocampal and cerebral cortical slices (Stein and Johnson, 2002, Wu et al., 2006). In the well validated APP23 transgenic mouse model, rather than amplifying disease, TTR over-expression suppressed both the neuropathologic and behavioral manifestations of AD (Buxbaum et al., 2008). In the same model, silencing the endogenous *ttr* gene accelerated disease pathogenesis (Buxbaum et al., 2008). In a recent study in mice we found that deletion of insulin receptor substrate 2 (Irs2) resulting in insulin resistance increased tau pathology as expected but paradoxically decreased amyloid pathology. We showed that this unexpected protection against plaque pathology was due to an increase in TTR expression (Killick et al., 2009), in line with a previous genome-wide expression study which found that increased TTR was one of the protective factors preventing transgenic mice with plaque pathology progressing to other pathological features of AD (Stein and Johnson, 2002).

In human AD a number of studies have reported reduced TTR levels in the cerebrospinal fluid (CSF) (Serot et al., 1997, Gloeckner et al., 2008, Hansson et al., 2009). Recent results from the MIRAGE study of AD families indicated that at least one TTR SNP (rs3764479) is associated with MRI documented hippocampal atrophy in AD patients and are consistent with a role for TTR in AD pathogenesis (Cuenco et al., 2011). Another recent study measured TTR levels in serum samples from 90 non demented

controls and 111 AD patients and observed significantly lower serum TTR levels in AD, suggesting it as possible peripheral biomarker for AD diagnosis in serum level (Han et al., 2011).

Whatever the mechanism, TTR is a prime candidate to influence Aβ pathology both directly and indirectly. The reasons for decreased plasma TTR levels in AD dementia subjects could be from altered morphology of the choroid plexus in AD with possible change of expression profile including TTR production and its transport into blood (Serot et al., 2000)]. Another possible explanation could be the down regulation of TTR expression in choroid plexus caused by activated β-secretase activity in AD with decreased sAβPPα (Stein et al., 2004).

TTR, ApoE and clusterin (ApoJ) are major Aβ-binding proteins in human CSF (Schwarzman et al., 1994, Strittmatter et al., 1993, Ghiso et al., 1993). In a recent report TTR was one of the 6 CSF biomarkers for AD describing six clinico-pathological stages from cognitive normalcy to mild dementia, including stages defined by increased risk of cognitive decline (Perrin et al., 2011). A recent review proposed a mechanism of TTR inhibition of Aβ toxicity, directly and indirectly (figure 6.1) (Li and Buxbaum, 2011). It is interesting that both clusterin and TTR proteins were identified and validated in human plasma, which differentiated fast and slow AD dementia progressors (Thambisetty et al., 2010, Velayudhan et al., 2012) .

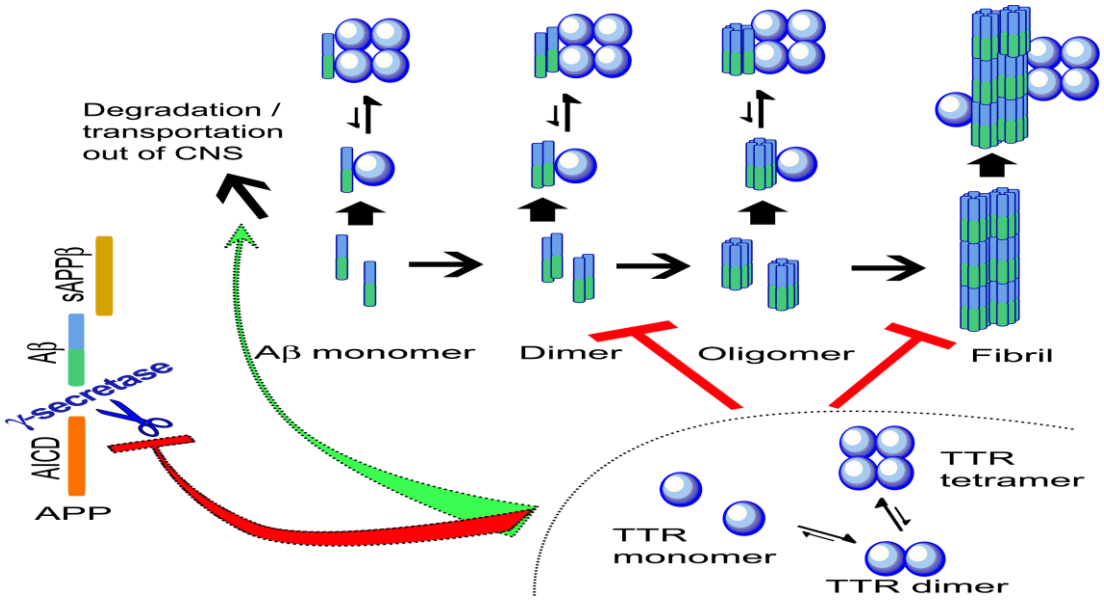
The study compared cases versus control and within patient cohort, fast versus slow decliners. Internal validation in an independent dataset was also performed. These studies have provided interesting candidates, however comparing AD with other neurodegenerative disorders for differential diagnosis is important and independent replications are critical for establishment of these markers.

The quest for AD plasma biomarkers is complicated by the fact that the rate of the progressive build-up of the pathology most likely varies from one individual to another and there is no precise pattern of disease onset across the AD population (Bazenet and Lovestone, 2012). The problem caused by this lack of synchronicity causes large

variability and can be partly addressed by examining large sample cohorts. Another caveat is that the normal population is also composed of individuals who may have biomarkers of AD pathology without the clinical manifestation of AD because of the long prodromal period (Bazenet and Lovestone, 2012).

There are number of challenges in order to translate the increasing optimism that these plasma proteins and other known and future candidate proteins could form a biomarker signature in blood specific for AD diagnosis. Platforms used in discovery are not necessarily the platforms used for either further validation/ replication or, more importantly, the platforms chosen for the clinical assays (Bazenet and Lovestone, 2012, Clark and Kodadek, 2013). Different technology, data processing and analyses performed can affect replication studies. For example, an MS-based method and an immunoassay trying to measure the same protein can produce discrepant findings. Different isoforms or post translational modification of proteins in plasma can give varied findings. It is also important to develop standardized protocols for plasma collection and of assays used for appropriate and meaningful comparison across studies, especially during the replication and validation phases (an issue also for CSF and imaging biomarkers) and finally, powerful and innovative bioinformatics tools will be needed to unravel multivariate plasma signatures (Bazenet and Lovestone, 2012).

Figure 6.1 - Proposed mechanisms of TTR inhibition of Aβ toxicity. TTR inhibition of Aβ aggregation (fibril formation) was reported by many groups and current evidence suggested that the binding is mediated by association of monomeric TTR to Aβ. It is also possible that TTR facilitates Aβ degradation directly or indirectly, transports of Aβ from CNS into serum (plasma sink hypothesis). TTR may also inhibit Aβ production by inhibition of γ-secretase cleavage **-** (Li and Buxbaum, 2011)



6.5 LIMITATION OF THE THESIS:

Most of the limitations have been discussed in detail in individual papers and studies. Some further issues raised by the reviewers are discussed as below;

For the olfaction study, participants with head injury, depression and other psychiatric disorders were ruled out, as these conditions can influence the olfactory function. Therefore, the generalizability of the study findings remains to be established. People with head injury have been studied to typically have anosmia, rarely regain normal olfactory ability and damage to olfaction-related brain structures observed in most such patients (Doty et al., 1997).

Olfactory dysfunction including identification deficits is well known in schizophrenia and described extensively in literature review (Nguyen et al., 2010, Turetsky et al., 2009). University of Pennsylvania Smell Identification Test (UPSIT) was tested in 131 patients with schizophrenia, 21 patients with major depression, 31 women with eating disorders along with 77 normal control subjects and olfactory identification deficits were observed only in patients with schizophrenia (Kopala et al., 1994).

Olfactory abilities in Major Depressive Disorder (MDD) have been less investigated, and available studies have provided inconsistent results. A recent study assessed odour recognition memory and odour identification in 12 mild MDD patients, 12 severe MDD

patients and 12 age and gender matched healthy normal controls. Data analyses revealed that Severe MDD patients performed significantly worse than Mild MDD patients and Normal controls on both tasks, with these last groups not differing significantly from one another (Zucco and Bollini, 2011). Depression was not associated with any major deficit in olfactory threshold or identification in a study including subjects with unipolar and bipolar depression (Swiecicki et al., 2009). In view of the inconsistent results it was decided to not include subjects with depression.

However smell identification test helps to distinguish people with dementia and depression in the elderly. A simple three-item test of olfactory identification differentiated AD dementia patients (n=40) from those with major depression (n=20) (Solomon et al., 1998). Another study used a German odor identification test in 20 patients with dementia due to AD, 20 with depressive disorder and 30 controls and found 100% sensitivity and 95% specificity with a score of 10/11 in differentiating AD from elderly with depression (Pentzek et al., 2007).

Depression and other psychiatric disorders were ruled out for both the cases and controls in the olfaction study. AD dementia patients were recruited from the clinical services within MHSOP and MHOA and the history of known depression or any other psychiatric illness was assessed by the referring psychiatric team. Patients with such a history were excluded.

However, for the elderly controls recruited from on-going studies at the centre, a depression questionnaire (Geriatric Depression Scale) was used to exclude depression.

For the control subjects, although the MMSE range was between 24-30, a history of any subjective and objective memory problems was also noted. MMSE has its limitation and with its ceiling effect for education and age and also cannot completely rule out people with mild cognitive impairment. However as all the control subjects were also participants in on-going biomarker studies at the Institute of Psychiatry centre and evaluated annually, I was able to confirm that they continued to remain stable as control subjects for the duration of this study and analysis.

The cut off range for MMSE decline was 2 points within 6 months. A number of investigators have previously reported average annual rate of change (ARC) of

approximately 2 to 4 points for the MMSE (Salmon et al., 1990, Behl et al., 2005, Galasko et al., 2000, Morris et al., 1993, Doody et al., 2001). These studies also supported reports that AD-associated drop in MMSE scores over time is non-linear. Mean cognitive decline of approximately 3 MMSE points per year has been described in classic AD (Morris et al., 1993). In a longitudinal study spanning 2 years among 686 patients with mild to moderate AD; 30% of patients had a decline that exceeded 3MMSEpoints per year, and 11% of patients had a mean (SD) decline of −4.57 (0.23) MMSE points per year, which was twice as fast as the mean of the whole cohort (Cortes et al, 2008). The limitation of use of MMSE rate of change, as with any clinical cognitive testing, is that it could be influenced by the day-to-day variability, behavioural problems, or medical illnesses (Sluimer et al., 2008). To minimise this, details of medical problems, medications and behaviour problems was noted at every assessment.

The short duration of follow up, 3 months may have under sampled individuals, whose disease progressed too slowly to be identified within the observation period or too rapidly to meet the eligibility criteria, leading to an underestimation of the heterogeneity in cognitive trajectories.

One of the limitations with the plasma proteins is that they can be influenced by diet, medications and medical conditions. To minimise the dietary effects, blood samples were collected following a 2 hour fast. The medical and treatment history were noted in detail at every assessment and included in the analysis. Decreased hepatic TTR expression is another possible cause of reduced TTR in AD but none of our AD dementia subjects had recorded liver dysfunction and additionally we did not find any differences in TTR levels of AD dementia subjects with and without thyroid dysfunction.

Some general issues relating to all the studies merit further discussion.
Firstly, MMSE score was used to measure cognitive decline in all the thesis studies. This was mostly as this was available for all the patients from different cohort and to increase the number of patient inclusion. As cognition can be influenced by behaviour, inter-current illness and medications etc, this was noted and assessed at every visit and considered during analysis. In the olfaction marker study, the patients and families account of symptoms worsening was also accounted in addition to loss of points on the

MMSE in the previous 6 months. The MMSE is the most widely used test as a measure of cognitive decline followed by ADAS-cog (Galasko et al., 1991, Behl et al., 2005, Schmidt et al., 2011). Paper 3 on the brain regions used both ADAS-cog and MMSE as measures of decline and associations were seen with both the cognitive measures. Also for the plasma markers, ADAS-cog was used for discovery phase to define the decliners and thereafter association of the markers were significant with the retrospective and prospective decliners defined by MMSE scores. Future studies should investigate these progression markers predictive of decline on other cognitive measures.

Secondly, all these subjects have not yet been observed throughout the duration of their illness. Therefore, the findings apply only to individuals with mild-moderate AD dementia observed up to 1 year of illness. It cannot be said that the predictive utility of these markers are equally strong in the second, third and later stages of the illness. Better understanding of models and mechanisms of rates of progression in AD would help clinical prognostication and also independent replication and validation of these markers in well -designed and well-controlled studies with larger cohorts essential for their research and clinical utility.

Finally, although the sample was drawn from AddNeuromed study for the biological markers i.e., plasma proteins and the brain regions, the subjects examined didn't overlap completely, so the sample size was not large enough to assess for common markers. However, the subjects assessed for plasma clusterin, who also had imaging data, showed association with smaller ERC (Thambisetty et al., 2010).

6.6 IMPLICATIONS AND FUTURE DIRECTIONS IN CLINICAL CONTEXT

Late-onset Alzheimer disease dementia is a clinically heterogeneous complex disease defined by progressively disabling cognitive impairment and the rate of the progressive build-up of the pathology most likely varies from one individual to another causing large variability (Bazenet and Lovestone, 2012, Yang et al., 2011). In the clinical setting, it is important to recognize disease heterogeneity and to understand the biologic variables involved for advancing diagnostic procedures, improving estimation of progression, and adapting treatment strategies (Schmidt et al., 2011). Non-invasive

markers of progression will also be of great importance to monitor therapeutic efficacy with disease-modifying treatments.

Tremendous progress has been made in the area of biomarkers, undoubtedly, others will emerge over time and despite the robustness of many of the biomarkers, such as CSF markers, multiple magnetic resonance (MR) biomarkers; temperoparietal hypometabolism on fluorodeoxyglucose-positron emission tomography (FDG-PET) or hypoperfusion on SPECT, and most recently amyloid PET imaging (McKhann et al., 2011, Jack et al., 2011), there remains interest in finding cheap, simple, and reliable alternatives, most especially those that could be easily collected, example blood biomarkers (O'Brien, 2013).

Dubois and colleagues, of the International Work Group (IWG) and the National Institute on Aging (NIA) and the Alzheimer's Association (AA), NIA/AA work groups support the diagnosis of AD prior to the onset of dementia and point to how best to integrate biomarkers into diagnostic criteria (Dubois et al., 2007, McKhann et al., 2011). It is hypothesized that amyloid biomarkers may be abnormal 10–20 years before the onset of symptoms, while abnormalities in biomarkers of neurodegeneration occur later (Jack et al., 2011). The presence of the biomarker represents a risk factor for progressing to AD in the future, however, the proportion of people who progress, the time frame for progression, and additional risk factors for progression are currently not fully defined (Cummings, 2012).

In a recent debate, Dubois, Gauthier and Cummings argue the need for revision of Alzheimer's diagnostic accuracy and many benefits to moving to a new diagnostic system, representing a fundamental paradigm shift, moving AD from a clinic-pathological to a clinic-biological entity and separating the diagnosis of Alzheimer's disease from Alzheimer's dementia (O'Brien, 2013). The new diagnostic criteria is supported by large body of research over 25 years, enhancing diagnostic specificity for the first time by using CSF and imaging markers to 'rule in' rather than simply rule out other disorders. However, it is also emphasized that these are research criteria and require validation and further investigation of clinical utility (Chiu and Brodaty, 2013).

Given a number of different AD biomarkers, it is inevitable that different combinations of test results can occur. At present, the data are insufficient to recommend a scheme that arbitrates among all different biomarker combinations. Further studies are needed to prioritize biomarkers and to determine their value and validity in practice and research settings (McKhann et al., 2011).

The body of work in the thesis adds evidence to the literature by validating the markers - olfactory identification, blood proteins (clusterin and transthyretin) and structural imaging (entorhinal cortical thickness) in that they are affected in AD compared to non-demented control subjects. The results from the plasma marker studies are believed to have wider implications for the identification of other amyloid chaperone proteins in plasma, both as putative AD biomarkers as well as drug targets of disease-modifying treatments. Entorhinal thickness as a predictive factor for further cognitive decline, which is also the brain region critical for the flow of olfactory information, implies that it would be informative to test these markers as combination markers in future studies for severity and for monitoring disease progression.

The thesis studies present interesting candidates; however, external replication is essential for establishing these markers. Future studies with enhanced collaborative work, standardised laboratory and radiological techniques, measurements and interpretation of results, are central for much-needed biomarker panels for improving prediction, diagnosis and monitoring of AD.

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APPENDIX

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Smell identification function as a severity and progression marker in Alzheimer's disease

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ABSTRACT

Background: Olfactory dysfunction, impaired smell identification in particular, is known as a diagnostic and a marker of conversion in Alzheimer's disease (AD). We aimed to evaluate the associations of olfactory identification impairments with cognition, illness severity, and progression in AD patients.

Methods: Fifty-seven outpatients with late onset mild to moderate AD and 24 elderly non-demented controls (NDC) were assessed, at baseline and after three months, for Mini-Mental State Examination (MMSE), University of Pennsylvania Smell Identification Test (UPSIT), and Bristol Activities of Daily Living and Neuropsychiatry Inventory. AD participants were classified as Rapid Cognitive Decliners (RCD) defined on *a priori* with a loss of $\geq$2 points in MMSE within the previous six months.

Results: AD participants had lower olfactory scores than NDC. RCD had lower olfaction scores compared with Non-Rapid Cognitive Decliners (NRCD). Although the baseline UPSIT scores were associated with baseline MMSE scores, it did not interact significantly with change in MMSE over the follow-up period. Using a median split for olfactory scores, the AD participants were classified as Rapid Olfactory Progressors (ROP) (UPSIT $\leq$ 15) and Slow Olfactory Progressors correlating significantly with RCD/NRCD groups. The ROP group with higher olfactory impairment indicated more symptomatic illness or severity, i.e. lower cognition, higher functional dependence, and presence of behavioral symptoms.

Conclusions: Our study supports association of smell identification function with cognition and its utility as an adjunct clinical measure to assess severity in AD. Further work, including larger longitudinal studies, is needed to explore its value in predicting AD progression.

Key words: smell identification test, dementia, Alzheimer's disease, olfaction, disease progression, biomarkers

Introduction

Alzheimer disease (AD) is a progressive and the commonest form of dementia. The course of AD is variable and factors that influence or predict progression are not well understood (Kraemer *et al.*, 1994; Marra *et al.*, 2000). The ability to differentiate rates of decline would help patients and caregivers plan for future, and physicians plan for appropriate treatment (Gauthier *et al.*, 2006). Disease progression is measured, most commonly, by change in cognition over time (Kraemer *et al.*, 1994; Marra *et al.*, 2000). However, clinical and neuropsychological measures may lack sensitivity to change, are subject to day-to-day variability, and are influenced by behavioral fluctuations and inter-current illness and medications (Sluimer *et al.*, 2008).

Olfactory dysfunction in general and impaired odor identification in particular have been reported in AD and are found to be occurring at early stages of the disease (Mesholam *et al.*, 1998). It has been indicated that involvement of the olfactory bulb and tract is one of the earliest events in the degenerative process on the central nervous system in AD (Christen-Zaech *et al.*, 2003) and also that tau pathology in the olfactory bulb increases with severity of AD (Attems *et al.*, 2005).

There are no published reports of smell identification in AD which have failed to find deficits relative to healthy elderly (Rahayel *et al.*, 2012). Smell identification tests have demonstrated

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high sensitivity and specificity for detecting Alzheimer's patients from controls (Morgan and Murphy, 2002). Olfactory dysfunction has been suggested to be included in the diagnostic criteria of AD (Foster *et al.*, 2008). However, a recent systematic review concludes that although there is evidence suggesting an association between decreased olfaction and AD, rigorously designed longitudinal cohort studies are necessary to clarify the value of olfactory identification testing in predicting the onset of AD (Sun *et al.*, 2012). A recent meta-analysis (Rahayel *et al.*, 2012) suggests that AD even more than Parkinson's disease (PD) affect more strongly smell identification and then smell detection, suggesting that AD patients are more strongly impaired on higher order olfactory tasks involving specific cognitive processes.

Studies have reported association between olfactory impairment and subsequent cognitive decline (Wilson *et al.*, 2006; 2007; Schubert *et al.*, 2008; Sohrabi *et al.*, 2012) and also been studied as a marker for predicting conversion from mild cognitive impairment (MCI) to AD (Devanand *et al.*, 2000; 2008).

However, there is little known about association of olfactory identification impairments and cognitive decline with illness progression in AD patients. In view of the common anatomical substrate for memory deficits and the olfactory function in AD, we hypothesized that olfactory identification ability at baseline would correlate with the cognitive ability and also predict altered cognitive function in a follow-up assessment. The specific questions examined by the current study were the following: (1) What is the difference between olfactory identification function between patients with mild to moderate AD and non-demented controls (NDC)? (2) Does olfactory function deteriorate with time period and do they differ for AD and NDC? (3) Is there any association between olfactory function and cognition in mild to moderate AD and can olfactory function predict future cognitive decline? (4) Is there any association between olfactory function and other non-cognitive symptoms in AD?

Subjects and methods

Subjects

AD participants: Late onset, mild to moderate AD participants (Mini-Mental State Examination (MMSE) score: 15–25) (n = 64) were recruited from Mental Health for Older Adults (MHOA) services of the South London and Maudsley (SLaM) NHS Foundation Trust and Mental Health Services for Older People (MHSOP), Leicestershire Partnership NHS Trust, United Kingdom. Diagnosis of probable late onset Alzheimer's disease was made according to the National Institute of Neurological and Communicative Disorders and Stroke and the Alzheimer's Disease and Related Disorders Association (NINCDS–ADRDA) criteria (McKhann *et al.*, 1984). The *exclusion criteria* were dementia other than AD; history of psychiatric disorder, including substance abuse; medical conditions that may alter cerebral functioning, or other conditions known to affect olfactory functioning (e.g. common cold, blocked nasal passages, polyps, etc.). Patients had either no history at all of cigarette smoking or had stopped smoking for 20 years or more. Informed consent or assent as appropriate was taken from all the patients. Participants received cholinesterase inhibitor therapy; either donepezil 5 mg/day (n = 21) or galantamine 8 mg/day (n = 15) for initial four weeks, which was then increased to 10 mg/day (donepezil) or 24 mg/day (galantamine) by their respective clinical teams, as per the local Trust policy. Some patients (n = 28) were not on cholinesterase inhibitors because of reasons such as cardiac contraindications, intolerance, patients not willing for therapy, or compliance issues.

Non-demented control patients

Eligible and interested NDCs were recruited from the on-going AD biomarker studies at the center for this olfaction study. As part of these biomarker studies, patients with AD and MCI, and NDCs were recruited and assessed as per standard protocol described previously (Thambisetty *et al.*, 2010; Velayudhan *et al.*, 2012). *Inclusion criteria*: MMSE score range between 24 and 30, Clinical Dementia Rating (CDR; Hughes *et al.*, 1982) scale score of 0, age 65 years or above, medication stable, and good general health. *Exclusion criteria*: Meet the Diagnostic and Statistical Manual of Mental Disorders (DSM)-IV criteria for dementia, significant neurological or psychiatric illness, significant unstable systematic illness, or organ failure.

Assessments

Baseline assessments were performed with a follow-up assessment after three months.

Assessment included cognitive testing with MMSE (Folstein *et al.*, 1975); non-cognitive symptoms using Neuropsychiatric Inventory (NPI; (Cummings *et al.*, 1994), daily activities with Bristol Activities of Daily Living Scale (BADL; Bucks *et al.*, 1996), and smell identification function using University of Pennsylvania Smell Identification Test (UPSIT; Doty *et al.*, 1984; 1995). UPSIT

is a standardized test of smell identification with good test–retest reliability (r = 0.95) and strong correlation with detailed olfactory threshold tests (r = 0.80; Doty *et al.*, 1984). This "scratch 'n sniff" olfactory test consists of four booklets containing ten odorants apiece; one odorant per page. The stimuli are embedded in 10–50-micron diameter microcapsules fixed in a proprietary binder and positioned on brown strips at the bottom of each page. Above each odorant strip is a multiple-choice question with four response alternatives for each item. The UPSIT has been used in British population to differentiate dementia patients from normal elderly British patients tested in their homes (Gray *et al.*, 2001) and as a treatment response marker (Velayudhan and Lovestone, 2009).

Patients were classified as AD Rapid Cognitive Decliners (RCD), based on a decline of ≥2 points at baseline in the previous six months of MMSE, a commonly used cognitive test to assess severity and decline in clinical settings (Behl *et al.*, 2005).

Using a median split, AD participants were further divided into two groups: those with more olfactory dysfunction, i.e. Rapid Olfactory Progressors (ROP; UPSIT ≤ 15) and Slow Olfactory Progressors (SOP), i.e. those with lesser impaired olfaction (UPSIT ≥ 16).

SPSS 20.0, STATA 10 and Excel 2010 were used for statistical analysis of the data and graphs. Comparisons were made on demographic information, clinical characteristics, and cognitive and behavioral test results with parametric (students *t*-tests) and nonparametric (Chi-square test, Mann–Whitney tests, Spearman rank correlations) statistics, as appropriate. Alpha level was set at 0.05; corrections for multiple comparisons were not made, given the exploratory nature of the study. Linear regression was performed with the MMSE scores over follow-up as the dependent variable and baseline UPSIT scores, age, baseline MMSE scores, duration of illness, gender, education, and follow-up time as predictive variables within the AD cohort.

Results

Sixty-four AD patients were eligible and agreed to participate in the study; however, 57 successfully completed both the baseline and follow-up assessments. Seven patients dropped out owing to developing stroke, being hospitalized, or moving to institutional care in a different locality. Of the 28 NDCs recruited, 24 completed the study and four dropped out owing to physical ill health. The study includes 57 AD patients and 24 NDCs who completed both baseline and follow-up assessments. None of the AD patients reported subjective impairment in olfaction. All participants were white Europeans, except for one male and a female in each group.

Comparison of AD patients and NDCs

The socio-demographic and clinical comparison between the AD patients and NDCs are as described in Table 1a. The NDCs were younger and had higher education. They scored higher on the baseline MMSE and UPSIT measures. The AD patients took longer to complete the UPSIT, and more AD patients had a family history of dementia. At follow-up, the MMSE and the UPSIT loss was not different from baseline within the groups (Table 1a, Figure 1). The patients who dropped out were similar to the patients in main data in their demographic and baseline data. The seven AD patients had a mean age of 84.6 years (±5.5 years), 12.8 years of education (±2.7 years), mean MMSE scores 21 (±4), and mean UPSIT scores 14.4 (±7.3). The four NDCs had a mean age of 77.5 years (±11.5 years), 15 years of education, mean MMSE scores 28.8 (±0.5) and mean UPSIT scores 26.5 (±11.8).

Comparison of rapid cognitive decliners and non-rapid cognitive decliners

As described above, the AD patients were classified as RCD (n = 28) and Non-Rapid Cognitive Decliners (NRCD; n = 29), based on a decline of ≥2 MMSE points in the previous six months at the baseline. The two groups were not different in age, gender, duration of illness, follow-up period, family history, or the number of patients on cholinesterase inhibitors therapy. However, the RCD had lower baseline MMSE (statistically significant) and UPSIT (statistically not significant) measures compared with NRCD (Table 1b, Figure 1; Mann–Whitney U test). The follow-up MMSE and UPSIT scores were low for RCD compared with NRCD (statistically significant) (Table 1b, Figure 1; Mann–Whitney U test).

The baseline UPSIT score correlated with both baseline and follow-up MMSE ($p < 0.01$, $r = 0.4$) and follow-up UPSIT scores ($p < 0.001$, $r = 0.6$). The UPSIT scores improved in AD patients on cholinesterase inhibitor treatment although this was not statistically significant (Figure 2).

UPSIT progressors

Using a median divide, the AD participants were further classified into ROP (UPSIT ≤ 15; n = 25) and SOP (UPSIT ≥ 16; n = 32). The two groups were similar in their socio-demographic data,

Table 1. Comparison of socio-demographic–clinical parameters between the groups. (a) AD patients and NDCs; (b) within AD cohort: rapid cognitive decliners (RCDs) and non-rapid cognitive decliners (NRCDs) and rapid olfactory progressors and slow olfactory progressors

	(a) AD PATIENTS AND NON-DEMENTED CONTROLS			(b) COMPARISONS WITHIN AD PATIENTS (n = 57)					
VARIABLES	AD (n = 57)	NDC (n = 24)	p-VALUE	RAPID COGNITIVE DECLINERS (n = 28)	NON-RAPID COGNITIVE DECLINERS (n = 29)	p-VALUE	RAPID OLFACTORY PROGRESSORS (n = 25)	SLOW OLFACTORY PROGRESSORS (n = 32)	p-VALUE
Female/male	35/22	14/10	NS[a]	16/12	19/10	NS[a]	17/8	18/14	NS[a]
Age (years)	81.4 (5.4)	77.3 (6.6)	<0.01*	80.6 (5.7)	82.1 (5.1)	NS*	81.9 (6.0)	80.9 (4.9)	NS*
Education	10.6 (1.4)	14.2 (4.7)	<0.001*	10.7 (1.5)	10.4 (1.3)	NS*	10.8 (1.5)	10.3 (1.2)	NS*
Duration of illness	27.8 (27.1)	n/a	n/a	27.5 (21.5)	28.1 (22.4)	NS*	27.3 (19.0)	28.1 (24.0)	NS*
MMSE baseline	21.6 (3.7)	29.1 (0.9)	<0.001*	20.1 (2.9)	23.0 (3.5)	0.001*	20.2 (3.1)	22.7 (3.6)	0.01*
MMSE FU	21.7 (3.9)	29.2 (0.7)	<0.001*	20.4 (3.5)	22.9 (4.0)	0.02*	19.7 (3.1)	23.3 (3.3)	0.001*
UPSIT baseline	16.1 (5.3)	28.6 (5.8)	<0.001*	15.0 (4.9)	17.2 (5.2)	0.07*	11.8 (2.2)	19.6 (4.1)	<0.001*
UPSIT FU	16.1 (5.4)	28.8 (5.9)	<0.001*	14.4 (5.3)	17.8 (5.2)	0.02*	13.4 (4.5)	18.3 (5.2)	0.001*
BADL baseline	9.3 (7.3)	n/a	n/a	9.5 (7.6)	9.1 (7.1)	NS*	13.5 (7.3)	6.0 (5.4)	<0.001*
BADL FU	9.6 (7.1)	n/a	n/a	9.4 (7.6)	9.9 (6.6)	NS*	12.5 (8.1)	7.0 (4.8)	<0.01*
NPI baseline	7.0 (9.9)	n/a	n/a	7.1 (9.2)	6.9 (10.8)	NS*	9.7 (11.2)	5.0 (8.6)	0.05*
NPI FU	5.5 (7.9)	n/a	n/a	5.2 (5.6)	5.7 (9.5)	NS*	7.4 (5.7)	4.2 (9.2)	<0.01*
Family history	16 (34%)	0%	0.001[a]	8 (28.6%)	8 (27.6%)	NS[a]	7 (28%)	9 (28%)	NS[a]
Follow-up in weeks	19.9 (10.1)	28.1 (11.9)	<0.01*	21.8 (13.2)	18.2 (5.4)	NS*	20.6 (11.3)	19.4 (9.3)	NS*
UPSIT time	26.7 (9.2)	18.4 (6.4)	<0.01*	26.9 (10.6)	26.6 (7.6)	NS*	27.4 (11.5)	26.2 (6.9)	NS*
CI Therapy	34 (59.6%)	n/a	n/a	14 (50%)	20 (69%)	NS[a]	16 (64%)	18 (56%)	NS[a]

Note: Values are mean (SD) or n (%); [a]calculated using the χ^2 test, [b]calculated using the t-test, *Wilcoxon paired test.
AD: Alzheimer's disease; NDC: non-demented controls; MMSE: Mini-Mental State Examination; UPSIT: University of Pennsylvania Smell Identification Test; CI: cholinesterase inhibitor; FU: follow-up; BADL: Bristol Activities of Daily Living; NPI: Neuropsychiatric Inventory; n/a, not applicable.

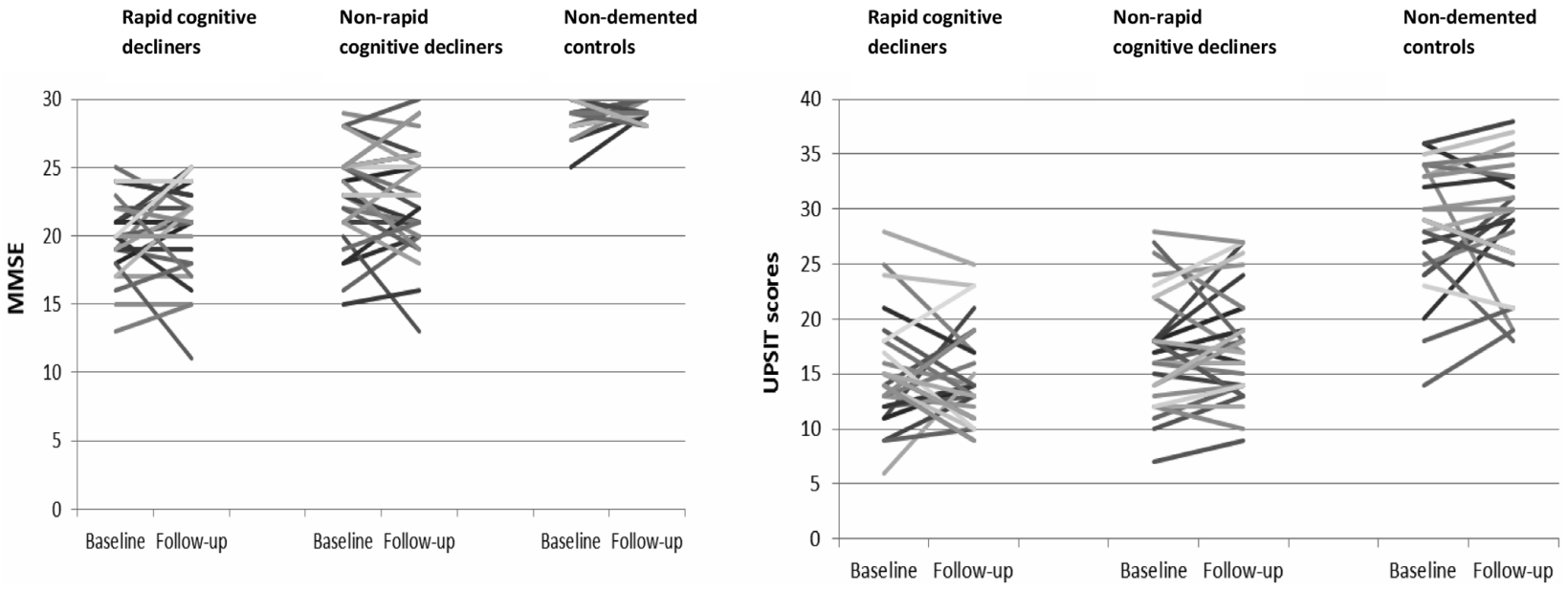

Figure 1. Line plot representation of baseline and follow-up MMSE and UPSIT scores between patients with Alzheimer's disease (RCD and NRCD) and non-demented controls.

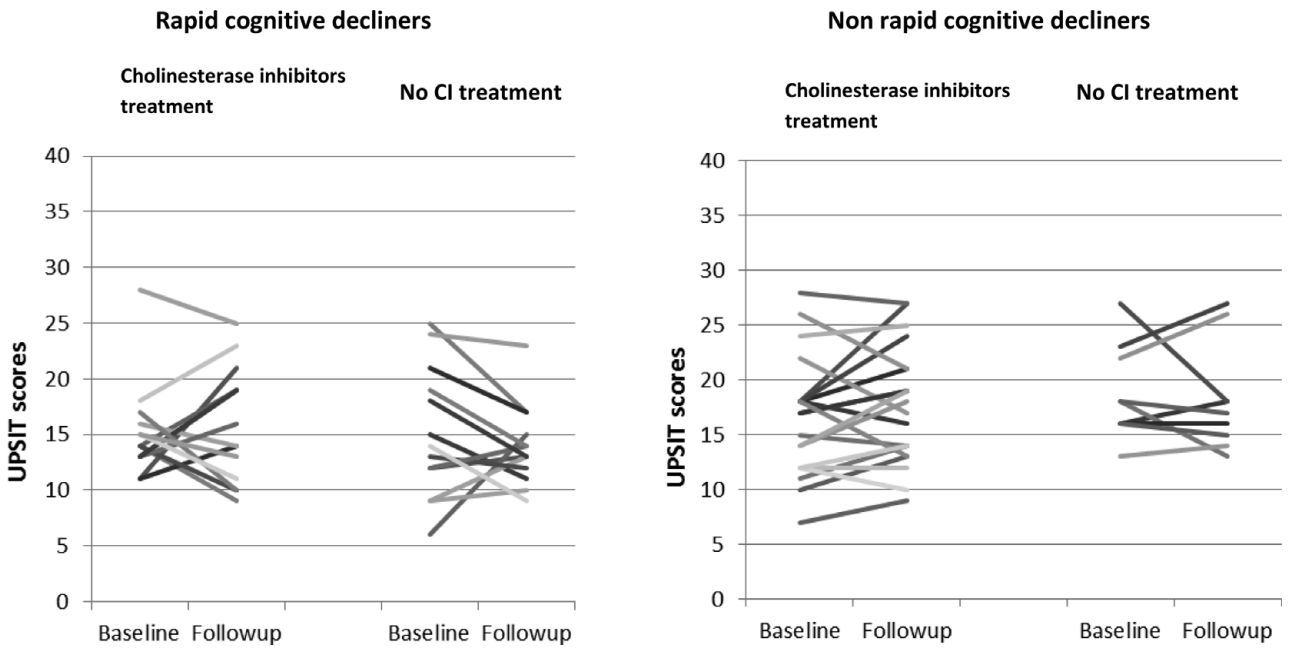

Figure 2. Line plots representation of UPSIT scores between RCD and NRCD patients with and without cholinesterase inhibitor (CI) treatment.

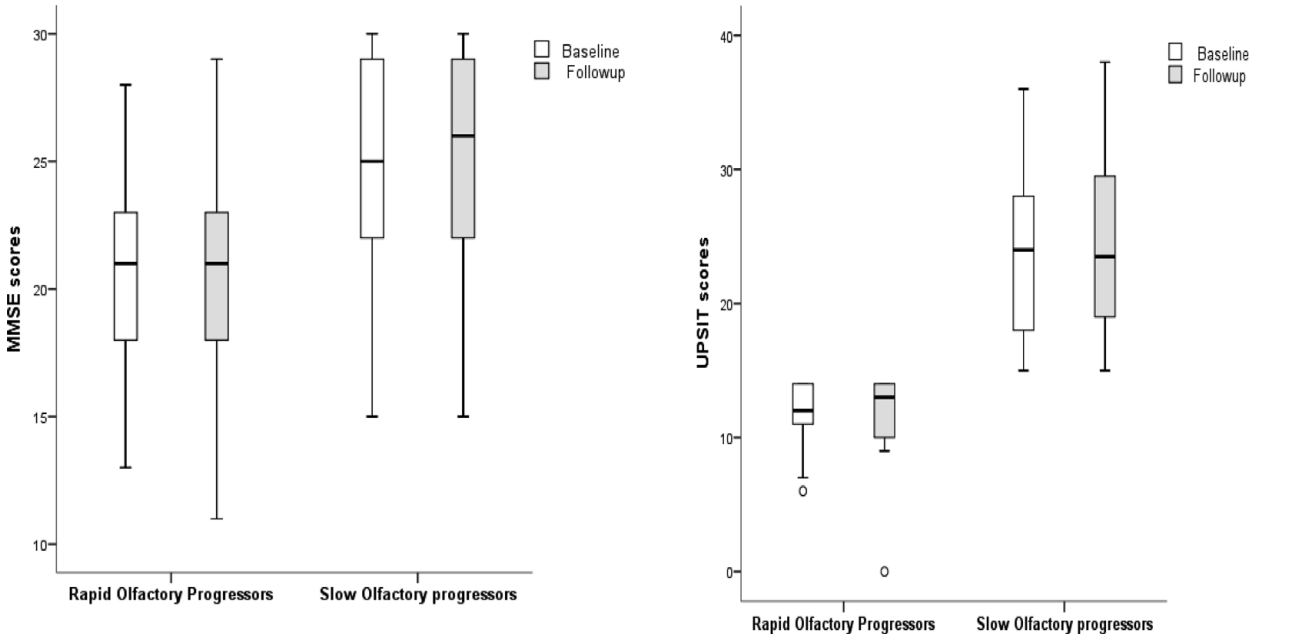

Figure 3. Box plot representation of baseline and follow-up MMSE and UPSIT scores between Rapid Olfactory Progressors and Slow Olfactory Progressors.

family history, follow-up time, and cholinesterase inhibitors treatment (Table 1b). However, the ROP patients had lower MMSE and lower UPSIT, implying more cognitive and olfactory impairment (Table 1b, Figure 3). They also had higher BADL and NPI scores, reflecting more functional deficits and more non-cognitive behavioral and psychological symptoms (Table 1b).

Linear regression analysis in unadjusted models showed baseline UPSIT scores, baseline MMSE scores, and gender as a better predictor factor for follow-up MMSE scores than variables such as age, gender, duration of illness, education, and follow-up time (Table 2). However, in the adjusted model, baseline UPSIT score was not significant (Table 2). Further, a linear mixed effects model using STATA 10 was fitted to investigate whether baseline UPSIT score was associated with overall MMSE levels measured in the two visits. The average baseline MMSE and the average change in the MMSE over

Table 2. Linear regression analysis with the follow-up MMSE scores as the dependent variable and baseline UPSIT scores, age, baseline MMSE scores, duration of illness, gender, follow-up in weeks, and cholinesterase therapy alternatively (Model 1) or simultaneously (Model 2) entered as predictive variables within the whole Alzheimer's disease sample

	R^2 (%)	β	95% CI	p-VALUE
Model 1				
UPSIT baseline	0.16	0.401	0.12, 0.5	<0.01*
Age in years	0.01	0.075	−0.14 0.25	0.578
Education	0.00	−0.017	−0.8, 0.74	0.903
Duration of illness	0.03	0.179	−0.02, 0.08	0.187
MMSE baseline	0.49	0.698	0.56, 0.99	<0.001*
Gender	0.08	−0.285	−4.4, −0.2	0.031*
Follow-up in weeks	0.01	−0.112	−0.15, 0.06	0.406
CI therapy	0.01	0.085	−1.47, 2.82	0.531
Model 2				
UPSIT baseline		0.165	−0.02, 0.28	0.098
MMSE baseline		0.619	0.47, 0.91	<0.001*
Gender		−0.205	−3.13, −0.17	0.030*

Note: R^2 (%): Percentage R^2 value for the overall model; *$p < 0.05$; CI: confidence interval; MMSE: Mini-Mental State Examination; UPSIT: University of Pennsylvania Smell Identification Test; CI: cholinesterase inhibitor.

follow-up time was calculated for all patients as a group (fixed effects) and subject-specific intercept and slope terms which reflected deviation from the group average (mixed linear effects) were calculated. The calculation included adjustment for follow-up time, age, disease duration, gender, and cholinesterase inhibitor treatment. A significant interaction between baseline UPSIT score and follow-up time (visit) was used to test the null hypothesis that there was no difference in the rate of cognitive decline, i.e. in slopes for different baseline UPSIT scores. We found that although baseline UPSIT was associated with lower MMSE at the baseline, the interaction between baseline USPIT and time was not significant ($p = 0.201$), indicating no association between baseline UPSIT and MMSE change (decline) over the two time points.

Discussion

Olfactory dysfunction has been studied as a diagnostic marker as well as a marker of conversion in AD. This is the first empirical attempt to investigate olfactory dysfunction as a severity and progression marker in AD. AD participants were deliberately chosen in the early stages (mild to moderate) of the disease so that there is little question of their ability to understand and perform the smell test. None of the AD participants had speech or language difficulties.

Olfaction in patients with AD and NDCs

AD patients had lower olfactory identification scores compared with NDCs, reported previously (Doty *et al.*, 1987; Richardson and Zucco, 1989; Serby *et al.*, 1991; Larsson *et al.*, 1999; Bahar-Fuchs *et al.*, 2011; Makowska *et al.*, 2011; Sun *et al.*, 2012). The median UPSIT scores in the study for the AD cohort and NDCs were similar to previous reports (Serby *et al.*, 1991; Gray *et al.*, 2001; Velayudhan and Lovestone, 2009; Li *et al.*, 2010). As a group there was no difference in the olfactory performance between the genders as reported previously (Westervelt *et al.*, 2007).

Olfaction and cognition

This study showed clear evidence of a relation of olfactory function with cognition: (a) Olfactory identification function was correlated with baseline and follow-up cognition. (b) The rapid and slow olfactory progressors correlated with the rapid and non-rapid cognitive decliners group.

Previous studies have found strong correlations between olfactory identification and cognitive performances (Serby *et al.*, 1991; Larsson *et al.*, 1999; Hidalgo *et al.*, 2011). Olfactory discrimination and identification have been more closely associated with higher cognitive functions and subsequent cognitive decline in community-dwelling elderly individuals (de Wijk and Cain, 1994; Wilson *et al.*, 2006; Sohrabi *et al.*, 2009). A large-scale study in older adults (n = 1920)

on the relationship between olfactory identification ability and general cognitive functioning (as measured by MMSE) indicated that olfactory dysfunction at baseline was significantly predictive of future cognitive impairment after five years (odds ratio (OR) = 6.62; confidence interval (CI) = 4.36–10.04; Schubert *et al.*, 2008). A strong association between cognitive functions and olfactory functioning has been reported and it has been concluded that compared with the ability to detect odors, identification of odors is more challenging, perhaps due to a lack of access to verbal or visual representations of odors (Richardson and Zucco, 1989). Similarly, Schab (1991) noted that odor identification might represent a semantic memory function. Some researchers suggest that olfactory identification is primarily predictive of memory decline (Swan and Carmelli, 2002). A recent meta-analysis (Rahayel *et al.*, 2012) suggests that AD affects more strongly odor identification and odor detection, suggesting AD patients are more strongly impaired on higher order olfactory tasks involving specific cognitive processes. In a functional magnetic resonance imaging (fMRI) study, the blood oxygen level-dependent (BOLD) signal at primary olfactory cortex (POC) was found to be weaker in AD than in healthy control participants (Wang *et al.*, 2010). At the lowest odorant concentration, the BOLD signals within POC, hippocampus, and insula significantly correlated with UPSIT, MMSE, and CDR scores, demonstrating that olfactory fMRI is sensitive to the AD-related olfactory and cognitive functional decline (Wang *et al.*, 2010).

Olfaction and non-cognitive symptoms

The present study showed that higher olfactory impairment was associated with more dependence in functional abilities at the baseline. Olfactory function has been associated with functional dependence previously in patients with MCI and normal elderly participants (Wilson *et al.*, 2007). A previous report from our cohort had shown that olfaction scores predicted improvement better than cognitive scores as indicated by global and functional improvement in AD patients receiving cholinesterase inhibitor therapy (Velayudhan and Lovestone, 2009).

Interestingly, the present study also found an association in the olfactory rapid decliners group with behavioral symptoms (NPI; Table 1b). This has not been reported previously. This could be, as most of the previous studies have focused, an association of olfaction with cognition. This needs to be explored further in future studies.

On the whole, the study results reflect that higher olfactory impairment is indicative of more symptomatic illness or severity, i.e. lower cognition, higher functional dependence, and presence of behavioral symptoms.

The baseline UPSIT scores predicted the follow-up MMSE in an unadjusted model, however, losing this effect in adjusted model with MMSE and gender. Further, a linear mixed effect model showed that although baseline UPSIT was associated with MMSE at the baseline, there was no association between baseline UPSIT and MMSE change (decline) over the two time points. This could have been influenced by the cholinesterase inhibitors therapy in some patients which influences UPSIT scores more than the cognitive scores (Velayudhan and Lovestone, 2009) and also the short follow-up period. Also, there were more females than males in the cohort, who perform better on the olfactory tasks than men (Murphy *et al.*, 2002; Mullol *et al.*, 2012).

Main limitations of the study are its small sample size and single point follow-up over a short duration. Longer follow-up with multiple point testing and assessments of cognitive, functional and behavioral changes, would be more informative of predictive ability of the olfactory function for illness progression in AD.

Gender was predictive of lower follow-up MMSE scores, with women losing more MMSE points over follow-up period. The possible explanation could be that women have a higher risk of developing AD above 80 years of age (Copeland *et al.*, 1999). The present AD cohort had a mean age of above 80 years (81.5 years), so the progression too must have been faster in women than in men.

A diminished sense of smell has practical implications in relation to AD, such as decreased appetite, with resultant weight loss and poor nutritional status. Other problems may be the inability to detect noxious odors such as gas and smoke. Both patient and family should be made aware of this deficit and the potential problems this may cause.

In conclusions, the study confirms associations of olfaction with cognition in mild to moderate AD and supports the utility of the smell identification function as an adjunct clinical measure to assess severity in AD. Further work, including larger longitudinal studies, is needed to explore its value in predicting AD progression.

Conflict of interest

None.

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Description of authors' roles

Latha Velayudhan designed the study, carried out the data collection, and performed statistical analysis and interpretation. Megan Pritchard contributed to the data collection. Petroula Proitsi contributed to the statistical analysis. Latha Velayudhan wrote the paper with contributions from all the authors. Latha Velayudhan had full access to all of the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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